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Ecology of the Tick-Borne Pathogen, Rickettsia Parkeri, in its Primary Vector, Amblyomma Maculatum, and Select Vertebrate Hosts

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Ecology of the tick-borne pathogen, *Rickettsia parkeri*, in its primary vector, *Amblyomma maculatum*, and select vertebrate hosts

By

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Rickettsia parkeri is a tick-borne microorganism, only recently recognized to cause disease in humans. The ecology of this disease is largely unknown, and was addressed through a series of laboratory and field investigations. Feeding behavior of immature stages of the primary vector, *Amblyomma maculatum*, on mammalian, avian, and reptilian host models was investigated. It was determined that this tick does not feed on anoles, and nymphs do display longer periods of attachment to and are significantly heavier having fed on cotton rats as compared with quail. Field surveys indicate evidence of exposure to spotted fever group rickettsiae in small mammals and farm-raised quail in Mississippi, but not in passerines. Results from experimental studies demonstrated that cotton rats become acutely infected with *R. parkeri*, but that quail do not show evidence of infection. Additionally, nymphal ticks were not able to acquire the organism from inoculated animals. Finally, a reverse line-blot assay was developed to identify sources of bloodmeal in archived, field-collected *A. maculatum* samples. This dissertation contributes important findings to our understanding of the ecology of *R. parkeri* and has implications for future work on the subject.

DEDICATION

Dedicated to my mother, Liliana Moraru,

a bright light in my life, a beautiful soul.

I carry you with me always.

T…c.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Between 1940 and 2004, at least 335 new or "emerging" infectious diseases circulated among humans (Jones et al. 2008). These can be defined as "infections that have newly appeared in a population or have existed but are rapidly increasing in incidence or geographic range" (Morse 1995). Just over half (54.3%) of these emerging disease events (as defined by the first case or cluster of cases) have been associated with bacteria and rickettsiae, with this number having increased because of emerging drug-resistant bacteria (Jones et al. 2008).

Tick-borne rickettsial organisms, such as the agent of Rocky Mountain spotted fever (RMSF) and that of Mediterranean spotted fever (MSF), may be pathogenic to humans, but several rickettsiae are non-pathogenic or of unknown pathogenicity, leading to confusion among physicians and diagnosticians (Goddard 2001). Those agents of non-pathogenic or unknown status, however, should be regarded with care; some rickettsiologists have cautioned that all rickettsiae isolated thus far should be considered potential pathogens (Raoult and Olson 1999). In fact, some rickettsiae that were originally thought to be non-pathogenic were ultimately found to cause disease (Paddock et al. 2004). A specific example of this incited the research herein.

In 2002, a man from Virginia presented to a clinic with fever, headache, myalgia, arthralgia, and general discomfort. In addition, there were multiple eschars (areas of

necrosis) on his legs. The case was subsequently reported when Paddock et al. (2004) diagnosed the illness as infection with *Rickettsia parkeri*, a tick-borne rickettsial agent first identified in 1937 by R. R. Parker (Parker 1939). A second human case was reported in 2006 from Mississippi (Finley et al. 2006) and a third in 2007 from Virginia (Whitman et al. 2007). Thus far, more than 20 suspected or confirmed cases of *R. parkeri* infection have been documented in the United States (Paddock et al. 2010). States from which cases have been reported in the literature are Virginia (2 confirmed), Mississippi (1 confirmed, 3 probable), Texas (1 confirmed, 2 probable), Florida (2 probable), Kentucky (1 confirmed), South Carolina (1 confirmed), Alabama (1 probable), and Maryland (1 confirmed) (Paddock et al. 2008, Cragun et al. 2010). Cases of infection with *R. parkeri* have also been documented in Latin America. Two cases in Uruguay were confirmed, and in Argentina two cases were confirmed with seven more suspected cases reported (Conti-Diaz et al. 2009, Romer et al. 2011). Confirmed cases throughout the Americas are shown in Figure 1. In fact, since recognizing *R. parkeri* as a cause of human disease, there has been some speculation that cases previously reported as RMSF, caused by *R. rickettsii*, were actually *R. parkeri* infections (Raoult and Paddock 2005).

Given that *R. parkeri* is now recognized as a pathogen, understanding its maintenance in nature would help evaluate risk of human disease and encourage a more targeted approach to controlling transmission to humans. The pathogen is carried by the tick species *Amblyomma maculatum*, *A. triste* and *A. tigrinum*, though *A. maculatum* appears to be the primary vector for transmission to humans in the United States (Parker 1939, Goddard and Norment 1983, Goddard and Norment 1986, Venzal et al. 2004, Silveira et al. 2007, Sumner et al. 2007, Cohen et al. 2009, Tomassone et al. 2010). In an

experimental setting, *A. americanum* has been shown to be a competent vector (Goddard 2003) and the agent has also been found in field-collected A. americanum (Cohen et al. 2009). *Amblyomma maculatum*, or the Gulf Coast tick, is known to feed on small animals as a larva or nymph, and on large mammals as an adult. However, the range of animals that may serve as hosts is wide and little is known concerning the role of specific vertebrate hosts in the endemic cycle of *R. parkeri*. Specifically, neither host preference nor feeding performance on specific host species is well defined for *A. maculatum*. This information could shed light on what animals may be exposed to *R. parkeri* and may be susceptible to infection. Ultimately, studies should be designed to evaluate the influence of key vertebrate hosts in the maintenance and transmission of *R. parkeri*, and, perhaps more importantly, what vertebrate species might be useful as sentinels for infection in nature. In addition, prevalence of both infected vertebrate hosts and ticks in nature would help gauge potential disease exposure to this pathogen. The overall objective of this dissertation was to fill gaps in our understanding of the role of various vertebrates in the natural history of *R. parkeri*.

The specific aims for this research were to:

1. Determine host preference and feeding success of *A. maculatum* larvae and nymphs when given cotton rats (*Sigmodon hispidus*), anoles (*Anolis carolinensis*), and northern bobwhite (*Colinus virginianus*) as potential hosts.
2. Evaluate susceptibility of cotton rats and northern bobwhite to infection with *R. parkeri* and their ability to subsequently transmit the bacterium to feeding *A. maculatum*.

a. Objective 1: Assess host susceptibility to *R. parkeri* infection by needle inoculation and ability of feeding *A. maculatum* larvae to acquire rickettsiae.
b. Objective 2: Attempt isolation of *R. parkeri* from needle inoculated cotton rats and quail and acquisition fed *A. maculatum* nymphs.

3. Estimate the prevalence of *R. parkeri* in wild rodents and birds collected from various sites in Mississippi.
4. Assess the utility of reverse line blot hybridization for bloodmeal analysis in archived field-collected adult *A. maculatum*.

Figure 1.1 Cases of *Rickettsia parkeri* infection in the Americas.

Shown are confirmed cases of *R. parkeri* infection in humans throughout the Americas. Additional probable cases have been reported from the same countries and states, but also include Florida and Alabama.

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CHAPTER II

LITERATURE REVIEW

Importance of vector/tick-borne diseases

Vector-borne diseases have shaped history and impacted both human and non-human animal health globally. It was at the end of the 19th century that arthropods were determined to be able to act as vectors of disease (Harwood and James 1979, Gubler 1998). Ticks are particularly important vectors of disease for most vertebrates, including humans. Ticks are associated with the highest diversity of pathogens than any other arthropod vector, harboring viruses, bacteria, rickettsiae, and protozoans (Jongejan and Uilenberg 2004).

Tick-borne diseases of veterinary and human medical importance occur worldwide, affecting human and non-human animal health. With events such as climate change and anthropogenic disturbances including international travel (Gratz 1999, Harvell et al. 2002), it seems likely that any geographic distinction between disease occurrences will be blurred. It is widely accepted that species diversity is highest in tropical regions of the world (Hillebrand 2004). This seems to hold true for parasite diversity as well (Poulin and Rohde 1997, Rohde and Heap 1998). There is a physiological tolerance level of individual species for specific climatic conditions (Currie et al. 2004); change in climate should then imply change in species richness (H-Acevedo and Currie 2003), including vector diversity (Githeko et al. 2000). Changes in climate

seem indeed to be intertwined with vector distributions. Mild winters in Sweden have been demonstrated to be correlated with tick distributions expanding north (Lindgren et al. 2000). In addition, life cycles seem to be accelerating, with seasonal peaks of tick activity occurring earlier in the year (Bormane et al. 2004).

Amblyomma maculatum as a vector

Ticks are important vectors of disease agents. Because of characteristics such as persistence when feeding, long lifespans, and high reproductive potential, they are efficient at maintaining and transmitting many pathogens. Not only do they have few predators (mainly birds and fire ants), they generally feed on more than one host throughout their life cycles (Harwood and James 1979, Wojcik et al. 2001). This allows for widespread dispersal of disease agents.

Rickettsia parkeri was first isolated from Gulf Coast ticks (*Amblyomma maculatum*) taken from cows in Texas (Parker 1939). Because *R. parkeri* has only recently become of epidemiological interest, little is known about where it is found geographically. A study published in 2007 suggested an 11-12% prevalence of the bacterium at sites in Florida and Mississippi (Sumner et al. 2007). More recently, ticks collected in Florida and Mississippi were found to be infected with *R. parkeri* at an average rate of 28%, with the highest prevalence of 40% seen in Jackson County, Mississippi (Paddock et al. 2010). In Virginia, 41.4% to 43.1% of ticks screened were positive for *R. parkeri* (Fornadel et al. 2011, Wright et al. 2011). Thirty percent of *A. maculatum* tested in Arkansas were positive for *R. parkeri* (Trout et al. 2010). A range of 20-33% prevalence was reported in *A. maculatum* sampled from various counties in North Carolina in 2009 and 2010 (Varela-Stokes et al. 2011). These high rates of

infection of *A. maculatum* with *R. parkeri* are in contrast with studies done concerning *R. rickettsii*. This agent is typically found at lower rates in its tick vectors – generally less than 5% (Burgdorfer et al. 1975, Linnemann et al. 1980, Gordon et al. 1984, McDade and Newhouse 1986, Walker and Fishbein 1991, Wikswo et al. 2008).

Rickettsia parkeri has also been detected in *A. americanum* adults but at much lower rates (Goddard and Norment 1986), and in *A. triste* in Uruguay (Silveira et al. 2007). *Amblyomma tigrinum* ticks collected from dogs in Bolivia were positive for *Rickettsia* spp.; a blood sample from one of the dogs tested positive for *R. parkeri*, and all dogs had circulating antibodies to rickettsiae (Tomassone et al. 2010). Several other tick species, including *A. ovale*, *A. aureolatum*, and *Rhipicephalus sanguineus*, have been shown to be infected with *R. parkeri* (Medeiros et al. 2011).

Amblyomma maculatum is also associated with other agents of disease. In 1997, adult *A. maculatum* were removed from a dog showing clinical signs of hepatozoonosis (Vincent-Johnson et al. 1997). It was subsequently found that both *A. maculatum* larvae and nymphs have the ability to acquire *Hepatozoon americanum* from infected dogs and that the protozoan is passed transtadially in the ticks (Mathew et al. 1998, Ewing et al. 2002b). In 2000, it was determined that *A. maculatum* has the potential to vector yet another agent of disease, *Ehrlichia (Cowdria) ruminantium*, the causative agent of heartwater (Mahan et al. 2000). Heartwater is a fatal disease of both domestic and wild ruminants in Sub-Saharan Africa and the Caribbean (Cowdry 1925, Perreau et al. 1980), and therefore, *A. maculatum*'s potential ability to act as a vector of this protozoan has important implications for the cattle industry in the United States.

History and characteristics of *A. maculatum*

The history of *Amblyomma maculatum* dates back to 1844, when C.L. Koch first documented the physical characteristics of an adult male. The author did not have a female specimen, but the male was noted to be from "Carolina" (Koch 1844, Mahan et al. 2000). In 1912, a paper was published with details on the physical characteristics, host associations, geographical distribution, and biology of the different life stages of *A. maculatum* (Hooker et al. 1912). The authors report the tick's distribution to be along the Gulf and southern Atlantic coasts of the United States, in contrast to most *Amblyomma* species which are established in tropical or subtropical parts of the world (Sonenshine 1991). While Hooker et al. also note the tick as occurring in areas of South and Central America, these specimens may have been confused with other *Amblyomma* species (discussed later). The same paper documented the "gotch ear" condition that this tick causes in certain host animals (Hooker et al. 1912). The tick came under scrutiny once it was implicated in facilitating screw worm infestations of livestock and therefore causing significant economic losses in the southern United States (Bishopp and Hixson 1936). Various authors over the years since then have continued to add to the body of knowledge concerning the Gulf Coast tick (Hixson 1940, Koch and Hair 1975, Gladney et al. 1977, Price 1980).

In 1939, R.R. Parker brought *A. maculatum* to light for what was to become arguably the most important reason. Parker isolated two strains of an "infectious agent" from *A. maculatum* collected from cattle in Liberty County, Texas. Experiments performed with this "rickettsia-like infectious agent" showed it to be mildly pathogenic to guinea pigs (Parker 1939). Although this was reported just before the start of World War

II, it was only until the turn of the twenty-first century that Parker's work and thus the tick became significant again (Paddock et al. 2004).

Details of *A. maculatum*'s history are complicated by confusion with closely related species, namely *A. triste*. In 1901, a French scientist documented a female *A. maculatum* from Paraguay and several others from Argentina, Chile, and Mexico (Neumann 1901). Those from Mexico were found on a lizard. Three from Buenos Aires were from a mammal (Neumann 1901). Lahille reported *A. maculatum* from Argentina from a dog, following Neumann's descriptions (Lahille 1905). These and many subsequent publications (Gedoelst 1903, Hunter and Hooker 1907, Cooley and Kohls 1944) likely made an error in claiming certain specimens to be *A. maculatum*. In 1956, Kohls published a paper documenting the error, claiming that no *A. maculatum* specimens analyzed by the author originated from any farther south than Colombia and Venezuela. The author also suggested that past "*A. maculatum*" examined from Argentina may have instead been *A. tigrinum*. The species *A. triste* was documented to differ from *A. maculatum* because of the presence of paired spurs along metatarsi II, III, and IV. Kohls also noted that *A. triste* differs by "the presence ventrally in both sexes of a small tubercle at the postero-interno angle of all festoons except the middle one" (Kohls 1956). Because such details can be easily overlooked, it is no wonder there was some confusion in differentiating between these species.

The genus *Amblyomma* and the subfamily it belongs to, Amblyomminae, have been through some taxonomic rearrangements. The Ixodidae family is divided into two major phyletic lines: Prostriata and Metastriata. The former is composed only of the genus *Ixodes*. The Metastriata includes the remaining ixodid ticks, classified into four

subfamilies: Amblyomminae, Haemaphysalinae, Hyalomminae, and Rhipicephalinae (Hoogstraal and Aeschlimann 1982). Some have referred to Amblyomminae as its own family, Amblyommidae (Siuda et al. 2006, Rymaszewska and Grenda 2008). This review, however, will treat the group as a subfamily. The Amblyomminae were originally thought to have evolved earliest, followed by the Haemaphysalinae (Sonenshine 1991). More recent research, however, suggests that the Ambylomminae are not monophyletic. Black et al. demonstrated this with work performed targeting the mitochondrial 16S rDNA gene (Black and Piesman 1994). The same paper shows *A. maculatum* to be most closely related to those *Haemaphysalis* species included (*H. cretica* and *H. leporispalustris*). Of the *Amblyomma* species in the study, *A. maculatum* is positioned closest to *A. hebraeum* and *A. variegatum* (Black and Piesman 1994). This finding is contradicted by research carried out with the 18S nuclear rDNA gene, which supports the original tree proposed by Hoogstraal and Aeschlimann, which shows the Amblyomminae to be monophyletic. This study shows that, of those *Amblyomma* species included, *A. maculatum* branched off first, followed by *A. tuberculatum* (Black et al. 1997). A more detailed phylogenetic tree based on the mitochondrial 16S rDNA gene shows *A. maculatum* to be most closely related to *A. triste*, followed by *A. tigrinum* (Nava et al. 2008). This validates, to a degree, the taxonomic confusion between these species during the early years of their study.

Because *A. maculatum* has been shown to carry *R. parkeri*, the geographic distribution of this tick is important in determining how widespread the rickettsial agent may be. This tick has been documented along the Gulf Coast of the United States as early as the beginning of the 20th century (Hooker 1908, Hooker et al. 1912) in states such as

Florida, Alabama, Mississippi, and Texas, but also northward into states including Georgia, North and South Carolina, Arkansas, Oklahoma, Kentucky, Virginia, and Delaware (Bishopp and Trembley 1945, Felz et al. 1996, Cilek and Olson 2000, Goldberg et al. 2002, Sumner et al. 2007); it is additionally found in certain countries in Central and South America and even in the West Indies (Bishopp and Hixson 1936, Bishopp and Trembley 1945, Estrada-Peña et al. 2005). While it has been documented in Arizona and California (Bishopp and Trembley 1945, Sumner et al. 2007), this may have been a result of translocation from endemic areas. In Mississippi in particular, it was found in 17 of the state's 82 counties (Goddard and Paddock 2005), though we have found evidence of this tick in other Mississippi counties as well (Ferrari et al. 2012). Within this state, it has been found many miles away from the Gulf Coast (Goddard and Norment 1983).

In addition to its distribution, *A. maculatum*'s life cycle is important in understanding the potential for spread of disease. For example, its locality suggests that it is dependent on high temperatures (Bishopp and Hixson 1936); however, its behavior suggests otherwise about humidity. Studies show that *A. maculatum* is more resistant to dessication than other ticks including *Ixodes scapularis*, *A. americanum*, and *Dermacentor variabilis* (Yoder et al. 2008). This is in accordance with its behavior: *A. maculatum* spends the warmest portion of the day questing and does not need to descend from vegetation to rehydrate (Yoder et al. 2008). This may be why it is found out in open fields, while most other ticks in the same areas are found in forests or at the forest edge (Goddard 1997). Generally, *A. maculatum* larvae are present and seek hosts predominantly from July through November; they can survive between two and five

months depending on environmental conditions; temperatures above 70°F and high relative humidity are optimal for host-seeking activity (Hixson 1937, 1940). A separate and distinct population of *A. maculatum* in Oklahoma and Kansas has larvae seeking hosts earlier in the year, between June and October (Barker et al. 2004, Broce and Dryden 2005). Larvae take an average 4.5 days to engorge on their host and from nine to twelve days to molt (Hooker et al. 1912, Hixson 1937, 1940, Price 1980). Nymphs are most abundant in late winter and spring when temperatures are again above 70°F (Hixson 1937, 1940). Again, the Oklahoma population seems different in that nymphs are found from June through October (Barker et al. 2004). Nymphs in general take slightly longer to engorge than larvae, averaging at 5 days and approximately 23 days to complete their molt (Hixson 1940, Price 1980). Adult Gulf Coast ticks are primarily host-seeking from mid-April to early October, with a peak in late summer (Hixson 1937, 1940, Goddard 2002); in Oklahoma, they are abundant from February until July (Barker et al. 2004). Once on a host, females engorge in 7-14 days and can weigh up to 1 gram once fully engorged. One female has been recorded to deposit up to 18 497 eggs (Bishopp and Hixson 1936). Males will stay on the host even after mating so that they can find other mates (Hooker et al. 1912). Though there is a distinct population in Oklahoma and these ticks display slightly different timing in their life cycle, there do not seem to be barriers preventing the different populations from mating (Ketchum et al. 2006).

Vertebrate hosts for *A. maculatum*

The larval and nymphal stages typically parasitize cotton rats, rabbits, foxes, white-footed mice, bobwhite quail, and meadowlarks (Peters 1936, Hixson 1940, Ellis 1955, Kellogg and Calpin 1971, Demarais et al. 1987, Clark and Durden 2002, Broce and

Dryden 2005). Both larvae and nymphs attach on the head and neck regions of their hosts (Hixson 1937). Hixson (1940) speculated that meadowlarks are the most important host for both immature stages of the tick. In the same vein, Price (1980) stated that birds seem to be the most important hosts for both larvae and nymphs. Studies of meadowlarks performed in Texas confirmed that they are indeed a good host for this tick, one study finding a minimum of 80% of the birds being infested (Teel et al. 1988, Teel et al. 1998). Northern bobwhites have also been identified as good hosts for *A. maculatum* immature stages (Peters 1936, Bishopp and Trembley 1945, Doster et al. 1980, Teel et al. 1998). A lark bunting was recently reported to be parasitized by *A. maculatum* (Robbins et al. 2010). Adult stages are known to feed on medium to large mammals such as cattle, sheep, deer, coyotes, raccoons, dogs, and humans (Hixson 1940, Cooley and Kohls 1944, Bishopp and Trembley 1945, Philip and White 1955, White 1955, Demarais et al. 1987, Goldberg et al. 2002). In particular, adults often feed inside the external ear of cattle, indirectly causing the ears to be swollen and droopy, a condition termed "gotch ear" (Bishopp and Hixson 1936, Bishopp and Trembley 1945, Broce and Dryden 2005, Edwards 2011, Edwards et al. 2011a); this has also been reported in goats (Edwards et al. 2011a). Koch and Hair (1975) performed experiments to determine how well larvae and nymphs fed on certain hosts. They found that larvae that had engorged on opossums and deer mice weighed less than those that fed on cotton rats, eastern woodrats, raccoons, black-tailed jackrabbits, and bobwhite quail. With the same potential hosts, engorged nymphs weighed more if they had fed on bobwhite quail, cotton rats, eastern woodrats, and black-tailed jackrabbits (Koch and Hair 1975). Whether or not each stage exhibits

any host preference, however, has not yet been evaluated. This information is important in helping to determine potential reservoir hosts for *R. parkeri*.

Epidemiological implications of vertebrate hosts

Identification of vertebrate hosts that act as reservoirs or amplifiers of vector-borne pathogens is important in understanding the epidemiology of associated diseases. The behavior and ecology of one animal host species inherently differs from that of another species, and may consequently have varying implications for any microorganisms these host species may harbor. For example, the ecological space that a species occupies presents any associated ticks with different opportunities than another host species might.

Vertebrate diversity plays an important role in the epidemiology of vector-borne diseases. A generalist vector by definition feeds on various animal host species. This may be beneficial or detrimental to proliferation of associated pathogens. From one perspective, a high diversity of vertebrate hosts may serve to dilute vector-borne pathogens in that no one animal species is being exceedingly exploited by the vector in question; this is termed the dilution effect (Norman et al. 1999). This is directly relevant for human exposure to vector-borne pathogens transmitted by vectors that rely on a variety of vertebrate hosts, where humans act as accidental hosts that are generally not used by the vector. This high diversity of potential hosts may make humans less likely sources of bloodmeals. Another possibility is that, for generalist vectors that have a wide variety of vertebrate bloodmeal sources, that vector and any pathogens it harbors may be preserved if local extinctions of vertebrate hosts occur (Ostfeld and Keesing 2000). That is, high diversity of vertebrates can protect humans and other animals against vector-

borne disease transmission, but it may also protect the vector and the disease agent from extinction.

In the case of Lyme disease, it was found that both diversity of vertebrate hosts and dominance of competent reservoir hosts are important contributors to the dilution effect (Buskirk and Ostfeld 1995, Schmidt and Ostfeld 2001). Avian diversity was determined to play a very significant role in human incidence of West Nile virus (Ostfeld 2009). It is essential to note, however, that animals that are not competent hosts for an infectious agent, but that are hosts for the tick vectors, may still be important in the ecology of the disease; these hosts have the ability to aid in maintaining the tick population. It has been suggested that an amplification effect may also occur, given more incompetent hosts, which will increase the vector population and therefore biting rates. This is, of course, much more complicated than is detailed here and is beyond the scope of this review.

Bloodmeal analysis in ticks

Analysis of host DNA in vectors has been performed using a variety of techniques. Early studies obtained bloodmeal source information by serological tests such as the precipitin test (Weitz 1956, Tempelis 1975, Washino and Tempelis 1983). These methods, while useful, often cannot identify hosts beyond the order or family level. With the advent of DNA sequencing and improvements in molecular techniques, studies have been able to refine the specificity of bloodmeal identification. DNA sequencing alone can be useful, though expensive (Kent 2009). Group-specific primers have also been developed and proved useful (Kent and Norris 2005); these may, however, amplify homologous regions of DNA. Mass spectrometry has also been utilized to identify

bloodmeal sources in mosquitoes (Rasgon 2008, Wickramasekara et al. 2008). Molecular techniques such as PCR-restriction fragment length polymorphism (RFLP) (Kirstein and Gray 1996), heteroduplex analysis (Richards et al. 2006, Simo et al. 2008), and real-time PCR (Hurk et al. 2007) have also been used to detect vertebrate host DNA (Kent 2009). Reverse line blot hybridization (RLBH), however, is less expensive and has been used in several studies analyzing the bloodmeal of ticks (Humair et al. 2007, Moran Cadenas et al. 2007, Kent 2009, Scott et al. 2012).

The RLBH technique was first developed to type group A streptococci using *emm* gene polymorphisms (Kaufhold et al. 1994). The technique was soon adopted for various applications in vector-borne diseases, including detection of different genospecies of tick-borne pathogens such as *Borrelia burgdorferi* (Rijpkema and Bruinink 1996, Kurtenbach et al. 2001) and identification of vertebrate hosts of blood-feeding arthropods (Kirstein and Gray 1996). The latter has been performed by targeting the 12S rRNA gene (Cadenas et al. 2007, Humair et al. 2007) and the 18S rRNA gene (Pichon et al. 2003, Pichon et al. 2005) of vertebrates. In addition, some have used a region of the *cytochrome b* gene as a target (Kirstein and Gray 1996).

Spotted fever group rickettsiae in vertebrates

There is an adequate foundation of literature demonstrating on what hosts *A. maculatum* is found, consequently providing sufficient evidence for pursuing investigations into the role of specific vertebrates in *R. parkeri* maintenance. Some spotted fever group (SFG) serological work has been done with both domestic and wild animals; however, because of the cross-reactivity in the SFG, these assays cannot be used to make species-specific conclusions. A study conducted in Mississippi found sera from a

variety of mammals to be positive (15/365) when tested against antigen from *R. rickettsii* (Norment et al. 1985). In Brazil, horses and dogs have been found with antibodies that react with *R. rickettsii* antigen (Sangioni et al. 2005). Also in Brazil, capybaras have been reported to have sera reactive with *R. bellii* and *R. parkeri* antigens (Pacheco et al. 2007). In Texas, jackrabbits were also found to harbor antibodies reactive with *R. rickettsii* (Henke et al. 1990). Cattle tested in Mississippi were found to have low antibody titers to SFG rickettsiae (Edwards et al. 2011b). In Brazil, one jaguar (n=10) was reported as having serum reacting strongest with *R. parkeri* antigen when tested against 6 different *Rickettsia* spp. Additionally, an *A. triste* from the same jaguar was PCR positive for *R. parkeri* (Widmer et al. 2011).

Original experimental infections conducted in the 1930s demonstrated that a febrile illness developed in guinea pigs when inoculated with *R. parkeri* (Parker 1939), and these findings were supported by later work (Philip and White 1955, Goddard 2003). It has also been demonstrated that infection in guinea pigs with living *R. rickettsii* offers protection against *R. parkeri* and vice versa. This was not the case when using formalin-killed vaccines of the organisms (Lackman et al. 1965). Experimental infection studies showed that opossums (*Didelphis aurita*) and cattle seroconverted when inoculated with *R. parkeri*. Some animals (2/6 calves and 1/2 opossums) also became transiently rickettsemic (Horta et al. 2010, Edwards et al. 2011b). Cattle (n=80) inoculated with *R. conorii* were reported to be rickettsemic for up to 32 days (Kelly et al. 1991). The longest rickettsemia observed after exposure to *R. rickettsii* in experimental conditions was 3 to 4 weeks, in opossums (Bozeman et al. 1967). Using inbred laboratory mice, details of a rickettsial infection have been characterized. A high dose (2.25 x 105) *R. conorii* was

observed to establish a disseminated endothelial infection in mice on dpi 1, followed by clinical signs by dpi 4 and death dpi 5 or 6. These mice developed meningoencephalitis due to vascular injury and interstitial pneumonia. A low dose (2.25 x 103) was shown to produce signs of illness in the mice by dpi 5 with full recovery by dpi 10 (Walker et al. 1994). Another study performed with inbred laboratory mice found *R. parkeri* to be most concentrated in the heart, lung, liver, and spleen tissues. Intradermally inoculated mice developed characteristics eschars, while intravenously inoculated mice exhibited facial edema and splenomegaly (Grasperge et al. 2012b). Dogs inoculated with *R. conorii* were demonstrated to develop transient rickettsemia up to dpi 10 (Kelly et al. 1992). In Louisiana, 13% of shelter dogs tested (12/93) were positive by PCR for *R. parkeri* (Grasperge et al. 2012a).

Tick vectors as reservoirs of rickettsiae

For some *Rickettsia* species, there are no recognized vertebrate reservoirs or amplifiers. This suggests that the arthropod vector maintains the rickettsiae in the ecosystem. Transovarial transmission has been demonstrated for several SFG rickettsiae including *R. parkeri* (Goddard 2003), *R. africae* (Kelly and Mason 1991), and *R. helvetica* (Burgdorfer et al. 1979). This characteristic implies the role of reservoir, as the arthropods are therefore maintaining the rickettsiae in their population. *Aponomma hydrosauri* is a tick feeding on reptilian hosts and has been identified as the vector and reservoir of *R. honei* (Stenos et al. 2003). In general, because *Rickettsia* spp. exhibit transovarial transmission in their arthropod vectors, they do not have the constraints of an obligate vertebrate host reservoir (Azad and Beard 1998).

There are aspects of arthropod-rickettsial interactions and interspecific competition within tick vectors that are not well understood. Studies performed with *D. andersoni* and *R. rickettsii* over several tick generations reported unexpectedly high mortality rates and reduced egg viability (Burgdorfer and Brinton 1975, Niebylski et al. 1999). In addition, once infected with *R. montana*, *D. variabilis* ticks were shown to be refractory to transovarial transmission with *R. rhipicephali* and vice versa (Macaluso et al. 2002), although coinfections have been documented at low rates (Carmichael and Fuerst 2006, Stromdahl et al. 2008, Wikswo et al. 2008, Varela-Stokes et al. 2011). With the largely unknown influences of tick-rickettsial interactions and interspecific competition, it is important to bear in mind the possibility of ticks acting not only as vectors of pathogens but also as reservoirs.

Characteristics of *Rickettsia* species

Rickettsiae are members of the Alpha-proteobacteria, a class of bacteria which gave rise to mitochondria sometime after 2 billion years ago (Sagan 1967, Madigan and Martinko 2006). The order Rickettsiales is currently divided into three families: Rickettsiaceae, Anaplasmataceae, and Holosporaceae (Azad et al. 2009). The family Rickettsiaceae is composed of the genera *Rickettsia* and *Orientia* (Dumler et al. 2001).

The *Rickettsia* are obligate intracellular parasites (Raoult and Olson 1999, Madigan and Martinko 2006). Though parasitic, they have retained many independent metabolic functions such as small molecule synthesis (those to be used in macromolecular synthesis) and electron transport phosphorylation. They are passed from one vertebrate to another by arthropod vectors, because they cannot survive long outside a host (Madigan and Martinko 2006). Confounding this are reports of aerosol

transmission of *R. rickettsii* (Saslaw and Carlisle 1966, Saslaw et al. 1966, Raoult and Olson 1999). Regardless, coevolution between the bacteria and their vectors has led to a strong relationship highlighted by proficient multiplication, transstadial and transovarial transmission, and long-term maintenance (Azad and Beard 1998).

Members of the genus *Rickettsia* are small, measuring about 0.7-1.0µm long by 0.3-0.5µm wide (Hackstadt 1996). They are gram-negative and therefore have typical inner and outer cell membranes separated by a layer of peptidoglycan. Their lipopolysaccharide layer has not been shown to have any endotoxic activity associated with it (Hackstadt 1996). Virulence factors identified include OmpA, OmpB, putative adhesin orthologs, and RickA (Pornwiroon et al. 2009). Although no type IV secretion system proteins have been identified to date in *R. parkeri*, they are known for all other *Rickettsia* genomes that have been analyzed; it is likely *R. parkeri* has the same system in place.

Traditionally, the genus *Rickettsia* has been divided into three groups: the typhus group, the scrub typhus group, and the spotted fever group (Roux and Raoult 2000). The typhus group was composed of *Rickettsia typhi*, *R. prowazekii*, and *R. canadensis* (Weiss and Moulder 1984). Only one species was placed in the scrub typhus group – *Orientia (Rickettsia) tsutsugamushi* (Tamura et al. 1995). The eventual dissolution of the scrub typhus group was based on prominent morphological differences and analysis of the 16S rRNA gene (Ohashi et al. 1995, Tamura et al. 1995). The remaining *Rickettsia* species made up the spotted fever group (Sekeyova et al. 2001). Eventually, alternate groupings were proposed, including an organization of four groups: the typhus group, the spotted fever group, an ancestral group, and a transitional group (Walker et al. 2008, Valbuena

and Walker 2009). New phylogenies are now based on molecular data more than on morphology and clinical presentation, as was historically used for rickettsiae.

The most well-defined groups are the typhus group (TG) and the spotted fever group (SFG), differentiated based on antigenicity of their lipopolysaccharides (Hackstadt 1996). *Rickettsia parkeri* has been placed in the SFG along with *R. rickettsii* and several other species that are of unknown pathogenicity (Walker et al. 2008). Members of this group are both transovarially and transstadially (between stages of ticks) transmitted in their tick vectors as well as via tick bite to their vertebrate hosts (Raoult and Olson 1999, Goddard 2003, Walker et al. 2008). Their ability to be transovarially and transstadially transmitted may be attributed to their propensity to infect the salivary glands, midgut, and ovaries of ticks (Goddard 2003, Valbuena and Walker 2009). Fournier et al. (1998) proposed that the SFG be further divided into three subgroups based on analysis of the *ompA* gene: the *R. conorii* complex and two other clusters. *Rickettsia parkeri* was found to be most closely related to *R. africae*, strain S, *R. sibirica*, and '*R. mongolotimonae*' (Fournier et al. 1998). In fact, in one study using restriction fragment length polymorphisms (RFLP), the authors were not able to differentiate between *R. parkeri* and *R. africae* (Eremeeva et al. 1994). Authors of another paper proposed combining *R. conorii*, *R.parkeri*, *R. rickettsii*, and *R. sibirica* together in one group, while *R. australis* and *R. akari* would be in the second group, and the remaining non-pathogenic species (*R. amblyommii*, *R. rhipicephali*, and *R. montana*) would be in the third group (Andersson et al. 1999). Recently, however, Apperson et al. suggested that *R. amblyommii* may actually also cause human disease (Apperson et al. 2008), thus calling into question the validity of classification based on perceived pathogenicity status. Yet another study, however,

evaluated taxonomic positions of these organisms and suggested that *R. conorii*, *R. parkeri*, *R. sibirica*, and *R. africae* belong in a phylogenetic sub-group together (Goddard 2009). In 2004, a new member was identified (Blair et al. 2004) and later added to the SFG, "*Candidatus* Rickettsia andeanae" (Jiang et al. 2005). The pathogenic status of this novel rickettsia is yet to be determined, but it has been detected in ticks, specifically *A. maculatum*, removed from humans (Paddock et al. 2010, Jiang et al. 2012). Its presence in the Gulf Coast tick may have important implications for the *A. maculatum*-*R. parkeri* system. This novel rickettsia occurs sympatrically with *R. parkeri* (Fornadel et al. 2011, Varela-Stokes et al. 2011) and coinfection in *A. maculatum* has been reported (Varela-Stokes et al. 2011, Ferrari et al. 2012).

The SFG is a unique group based on both genetics and characteristics. Most rickettsial species currently included in this group are associated with ticks (Walker et al. 2008); the remaining species are *R. akari* and *R. felis*, transmitted by mites and fleas, respectively (Raoult and Olson 1999). These include *R. rickettsii*, *R. parkeri*, *R. amblyommii*, *R. rhipicephali*, *R. montanensis*, and *R. peacockii*, among others (Walker et al. 2008). Phylogenetically, members of the SFG consistently cluster separately from the TG. Species in this group have a genome between 1200 and 1400 kb, with few exceptions (Roux and Raoult 1993, Hackstadt 1996). The guanine+cytosine content of their DNA is typically higher than that of TG members – 32-33% as opposed to 29-30% (Hackstadt 1996). Genes of interest (mainly for species differentiation) are the *17kDa* (surface antigen gene), *gltA* (citrate synthase gene), *rompA* (rickettsial outer membrane protein A gene), and *rompB* (rickettsial outer membrane protein B gene) (Roux et al. 1997, Fournier et al. 1998, Roux and Raoult 2000). *Rickettsia parkeri* groups

phylogenetically with *R. africae* and strain S using *rompB* as a target (Roux and Raoult 2000). Considering *gltA*, *R. parkeri* is most similar to *R. sibirica*, '*R. mongolotimonae*', '*R. slovaca*', strain S, *R. conorii*, Astrakhan fever rickettsia, *R. africae*, and Thai tick typhus rickettsia (Roux et al. 1997). Targeting the 17 kDa antigen gene, *R. parkeri* groups closely with *R. rickettsii* and *R. conorii* (Stenos et al. 1998). Of those tested, all SFG rickettsiae react with monoclonal antibodies to the lipopolysaccharide-like antigen from *R. rickettsii* (Anacker et al. 1987). Indeed, there are high rates of serological cross-reactivity within all groups of rickettsiae (Parker 1939, Valbuena and Walker 2009).

Pathogenesis of rickettsiae

Rickettsial agents, and specifically most members of the SFG, are obligate intracellular bacteria found in a variety of ticks. Many SFG rickettsiae are carried by ixodid ticks, but not all are reported agents of disease (Azad and Beard 1998, Raoult and Olson 1999). Those that are, preferentially infect endothelial cells and need to expend energy to do so (Moulder 1985). To enter into a host cell, rickettsiae attach to the protein-dependent receptor Ku70 on the host cell membrane; binding is achieved using the outer membrane protein (Omp) B, found on the surface of all *Rickettsia* species, and results in recruitment of further Ku70 receptors to the host cell membrane (Walker 2007). The OmpA protein has also been recognized as important for *R. rickettsii* in adhering to and entering host cells (Li and Walker 1998); this and the OmpB protein have been identified in *R. parkeri* (Pornwiroon et al. 2009). On the other hand, *R. peacockii*, which is closely related to *R. rickettsii*, cannot synthesize the OmpA protein due to premature stop codons in the *ompA* gene (Baldridge et al. 2004). This possibly relates to the nonpathogenic status of *R. peacockii* and supports the idea that OmpA is important in cell-to-cell spread

of pathogenic rickettsiae. SFG rickettsiae use their own metabolic activity to enter host cells by provoking cytoskeletal rearrangements and recruitment of Arp2/3 to polymerize actin in the host cell such that the rickettsiae can enter, move within, and exit the cell (Walker 2007). A recent study determined that *R. parkeri* enters arthropod cells by pathways involving Wiskott-Aldrich syndrome protein (WASP)-family verprolin-homologous protein (WAVE) nucleation-promoting factor and Arp2/3 complexes, leading to actin nucleation (Reed et al. 2012). Inside, SFG rickettsiae quickly escape the phagosome, possibly by way of phospholipase A2 (Walker et al. 2003) or phospholipase D (Renesto et al. 2003) or a combination of the two, and enter the cytosol where they highjack nutrients, energy, and components for growth (Walker et al. 2003).

Characteristic of SFG rickettsiae, replication is possible not only in the cytoplasm, but also within the nucleus (Raoult and Roux 1997). They acquire metabolic energy by coupling ADP phosphorylation to the oxidation of glutamate, using ATP from the host (Moulder 1985). Inside host cell cytoplasm, the bacteria replicate by binary fission, filling the cell until it lyses (Madigan and Martinko 2006), releasing anywhere from 100 to 1000 new infectious units per host cell (Moulder 1985). Peak intracellular numbers of rickettsiae are achieved 3 to 5 days after infection (Moulder 1985). Lysis of the host cell may be due to simple restrictions of physical space, or it may be a result of the activity of phospholipase A, an enzyme implicated in entry of *R. prowazekii* and *R. rickettsii* (Moulder 1985). SFG rickettsiae also spread from one host cell to another via an actin-based motility, much like *Listeria* and *Shigella* species (Hackstadt 1998). Indeed, the ability to form actin tails has been reported as characteristic of SFG rickettsiae, including *R. parkeri*; this is not the case for TG rickettsiae (Heinzen et al. 1993). To be

immediately toxic, however, TG rickettsiae need to attach to host cells and release phospholipase A (Moulder 1985); this has not been documented for SFG rickettsiae (Hackstadt 1998).

History of *R. parkeri* and current knowledge

In 1939, a man named R. R. Parker published a report documenting isolation of a rickettsia from Gulf Coast ticks (*Amblyomma maculatum*) collected in Texas (Parker 1939). The organism was determined to cause mild disease for some laboratory animals including guinea pigs, monkeys, rabbits, white rats, and especially Sawatch meadow mice (*Microtus pennsylvanicus modestus*). The organism was referred to as "the maculatum agent" and the disease it caused was termed "maculatum infection" (Parker 1939). It was subsequently isolated from *A. maculatum* collected in Georgia in 1938 and in Mississippi in 1948 and 1955 (Lackman et al. 1949, Philip and White 1955). Eventually, the rickettsia adopted the name of its discoverer, becoming *R. parkeri* (Lackman et al. 1949, Lackman et al. 1965).

Almost seven decades after its first isolation, *R. parkeri* was implicated as a human pathogen. In August of 2002, a man presented to a clinic in Virginia with a fever, mild headache, myalgia and arthralgia, and general malaise. His lower extremities also had multiple eschars, or small areas of necrosis. He was unresponsive to amoxicillin-clavulanic acid and cephalexin. As a result of extensive laboratory tests, the patient was diagnosed with rickettsialpox (caused by *R. akari* and transmitted by mites) and treated with doxycycline. Within two days, the fever, arthralgias, and myalgias had resolved. Biopsy and serum specimens had been sent to the Centers for Disease Control and Prevention (CDC; Atlanta, GA) during the patient evaluation. The patient's serum was

determined to be reactive with both *R. rickettsii* and *R. akari*. DNA analysis determined the causative agent to be *R. parkeri* (Paddock et al. 2004).

Since the first diagnosis of disease due to *R. parkeri*, there have been about twenty additional human cases reported. The second documented case occurred in Mississippi (Finley et al. 2006), and the third report was of a man in Virginia (Whitman et al. 2007). Since then, probable and confirmed cases have been reported throughout the Southeastern United States (Figure 1). Cases of *R. parkeri* rickettsiosis have also been identified in parts of Latin America including Argentina (Romer et al. 2011) and Uruguay (Conti-Diaz et al. 2009).

Currently, *R. parkeri* is understood to be most closely related to *R. africae*, Strain S, *R. sibirica*, and *R. conorii* (Fournier et al. 1998, Andersson et al. 1999, Roux and Raoult 2000, Goddard 2009). There are four *Alu*I sites in the citrate synthase gene, and the *RsaI* and *PstI* profiles in the 190kDA gene for *R. parkeri* and *R. africae* are identical (Eremeeva et al. 1994). *Rickettsia parkeri*'s *Rsa*I band pattern in the 120kDA gene is identical to that of *R. africae* and *R. sibirica* (Eremeeva et al. 1994). These genetic similarities are mirrored in the clinical characteristics of human infection with *R. parkeri*, *R. africae*, and *R. conorii*.

Clinical aspects of *R. parkeri* rickettsiosis

Though first isolated in 1937, *R. parkeri* was only recognized as a cause of human disease in 2002 (Paddock et al. 2004). Whether or not *R. parkeri* also prefers to infect endothelial cells is not known; however, there is circumstantial evidence for this cell tropism in human case descriptions (Paddock et al. 2004, Walker 2007). The first case report of *R. parkeri* infection in a human described perivascular infiltrates containing

rickettsia-infected cells, suggesting endothelial involvement (Paddock et al. 2004). In addition, *R. parkeri* infection is clinically similar to African tick bite fever (*R. africae*), boutonneuse fever (*R. conorii*), and Rocky Mountain spotted fever (*R. rickettsii*), the agents of which also infect endothelial cells (Raoult and Paddock 2005, Valbuena and Walker 2009). In fact, in 2005, serum samples from some cases diagnosed as RMSF were shown to react with *R. parkeri* antigens suggesting that *R. parkeri* causes mild forms of an RMSF-like disease (Raoult and Paddock 2005).

Observations of disease caused by *R. parkeri* were originally made in studies concerning guinea pigs. They were found to develop a mild fever and, in the case of the males, a swollen and pink scrotum (Parker 1939, Lackman et al. 1949, Goddard 2003). The fever, if present, was found to last between one and four days, while edema and redness in the scrotum persisted from one up to eight days (Parker 1939). Rabbits injected with the "maculatum disease" agent have not shown clinical signs of infection and show low antibody response (Lackman et al. 1949).

In humans, infection with *R. parkeri* is characterized by fever and an eschar at the bite site (Goddard 2004, Paddock et al. 2004) and the disease has been variously referred to as maculatum disease, American boutonneuse fever, or simply *Rickettsia parkeri* rickettsiosis (Goddard 2004). Human infection occurs as a result of rickettsiae multiplying specifically at the site of tick attachment (Valbuena and Walker 2009). The eschar is of particular interest as it is a clear distinction between *R. parkeri* and *R. rickettsii* infection (RMSF cases have not reported eschars). Additionally, RMSF patients may present with a petechial rash, while patients with *R. parkeri* infection do not and instead may have rashes characterized by vesicles or pustules (Paddock et al. 2008,

Walker et al. 2008). Of the confirmed and probable cases to date, thirteen patients became sick between late July and early September. All of these patients had low to moderate fevers that lasted from two to eleven days. Fourteen cases were associated with eschars, thirteen patients had rashes, and ten experienced arthralgia (Paddock et al. 2008, Cragun et al. 2010). Lymphadenopathy has also been associated with some cases (Paddock et al. 2008). Cases have presented similar to the initial case, but have ranged in severity suggesting variable levels of virulence in different strains of *R. parkeri* (Paddock et al. 2008).

Gaps in current knowledge of *R. parkeri* ecology

Up to this point, studies of wildlife hosts for *A. maculatum* as potential amplifiers or reservoirs of *R. parkeri* have not been reported in the literature. Some work has been conducted with cattle (Edwards et al. 2011b), mice (Grasperge et al. 2012a), guinea pigs (Goddard 2003), and, in South America, opossums (Horta et al. 2010). This has not, however, addressed potential or actual hosts for immature stages of *A. maculatum*. Although there is documentation of hosts for all stages of *A. maculatum* (Hixson 1937), the extent to which this tick feeds upon each species has only been partially addressed (Koch and Hair 1975). As of yet, such information has not been complemented by laboratory experimental infections with *R. parkeri* in these animals. The dissertation presented here aims to address these issues.

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CHAPTER III

OBSERVATIONS ON HOST PREFERENCE AND FEEDING SUCCESS OF IMMATURE *AMBLYOMMA MACULATUM* (ACARI: IXODIDAE)

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Abstract

Amblyomma maculatum Koch, 1844 is the primary vector in the United States for *Rickettsia parkeri*, an emerging human pathogen, as well as *Hepatozoon americanum*, a protozoan causing disease in canines. We evaluated the host preference and feeding success of immature *A. maculatum* for three potential host species, the Carolina anole (*Anolis carolinensis*), the Bobwhite quail (*Colinus virginianus*), and the Hispid cotton rat (*Sigmodon hispidus*). To determine host preference, ticks were given an option of two different hosts at a time. No ticks fed on anoles in the host preference study and no significant difference in preference could be determined for rats compared to quail. In a separate experiment to study feeding success, we placed ticks directly onto ten individual

animals of these same species. No ticks fed on anoles. Larvae did not statistically differ in number of days to engorge when feeding on rats (5.7 d) compared to quail (5.6 d). Nymphs, however, took significantly longer to engorge on rats (8.2 d) than on quail (7 d). Engorged larvae from rats and quail were not statistically different in weight, while nymphs that engorged on rats were significantly heavier (15.8 mg) than those from quail (13.2 mg). Engorged larvae and nymphs did not significantly differ in their molting success between hosts. Results of this study suggest that anoles were not good hosts for immature stages of *A. maculatum*. No clear host preference was identified for quail or cotton rats, although differences in time-to-engorgement and engorged specimen weights were noted.

Keywords *Amblyomma maculatum*, feeding, hosts, cotton rat, quail

The Gulf Coast tick, *Amblyomma maculatum*, is an ixodid tick found throughout the southeastern United States and in some countries in Central and South America. Historical studies from over 50 years ago report that the immature stages feed on small mammals and ground-dwelling birds (Ellis, 1955, Peters, 1936, Hixson, 1940). In general, this has included mammals such as cotton rats and rabbits, and birds such as the eastern meadowlark and bobwhite quail. Adults are found on larger mammals such as deer and cattle (Cooley & Kohls, 1944, Hixson, 1940). A previous comparison of avian and mammalian host species for immature *A. maculatum* found that significant differences existed in the feeding success of these ticks (Koch & Hair, 1975). For example, engorged larvae and molted nymphs tended to have a greater weight, and earlier drop off time when feeding on quail, whereas nymphal ticks were significantly heavier as engorged nymphs and molted adults after having fed on cotton rats, compared to quail

(Koch & Hair, 1975). In all cases, opossums were poor hosts and tick feeding success on other species tested (Eastern woodrat, black-tailed jack rabbit, raccoon, and deer mouse) was variable. Reptiles were not evaluated in the Koch and Hair study (Koch & Hair, 1975), and, in general, have not been examined very closely in tick ecology studies. However, although *A. maculatum* has not been documented from reptiles, other ixodid tick species have; for example, *Ixodes scapularis* immature stages will feed on skinks and other reptiles (Apperson *et al.*, 1993, Oliver *et al.*, 1993). Under experimental conditions, however, larvae have been found to prefer and more successfully feed on white mice as compared to skinks (James & Oliver, 1990).

Amblyomma maculatum is known to bite humans and is a vector of the recently recognized human pathogen, *Rickettsia parkeri* (Goddard, 2002, Parker, 1939, Paddock *et al.*, 2004) and of the canine pathogen, *Hepatozoon americanum* (Ewing *et al.*, 2002b, Kocan *et al.*, 2000). It is also an experimentally competent vector of *Ehrlichia ruminantium*, the causative agent of heartwater, a foreign animal disease in the United States (Uilenberg, 1982). This tick's wide host range and vector potential make the ecology and epidemiology of any pathogens associated with it more complex, meriting studies of the vector-host-pathogen system. Furthermore, although the number of studies involving *R. parkeri* in particular has recently increased, none have, to our knowledge, specifically evaluated the natural history of the organism in *A. maculatum*. While ticks themselves are the main reservoirs of many *Rickettsia* spp., vertebrate hosts such as rodents and larger mammals may also play a role as amplifiers or additional reservoirs. For example, in the United States, the meadow vole (*Microtus pennsylvanicus*) has been implicated as an amplifier of *R. rickettsii*, while in Brazil the capybara and opossums are

thought to play that role (Labruna, 2009, Souza *et al.*, 2009, Bozeman *et al.*, 1967, Burgdorfer, 1988). It is unknown, however, what role vertebrate hosts used by *A. maculatum* have in the natural maintenance of *R. parkeri*. Thus, revisiting the basic ecology of *A. maculatum* would be a step towards understanding the epidemiology of *R. parkeri*.

This study was performed to better understand potential and actual hosts of *A. maculatum*. We chose two known hosts for *A. maculatum* from two Classes (Aves and Mammalia), and a third host from a Class (Reptilia) not known to be utilized by this tick species. Our results help elucidate the role of these representative species in the life cycle of *A. maculatum*, specifically for immature stages of this tick.

Materials and Methods

All *A. maculatum* larvae and nymphs used in this study were obtained from a laboratory-reared colony at Texas A&M University. Ticks were kept in humidity chambers at 90% relative humidity (RH) until used in the study within two months of receipt from Texas A&M University. Three potential host species, each representing a different Class of the Phylum Chordata, were used for both studies: cotton rats (*Sigmodon hispidus*; Harlan Laboratories, Indianapolis, IN), bobwhite quail (*Colinus virginianus*; Pollard Quail Farm, Mathiston, MS), and Carolina anoles (*Anolis carolinensis*; Carolina Biological Supply, Burlington, NC). For the duration of each trial, individual animals were housed in wire cages positioned on blocks over trays of water. The animals were given food and water *ad libitum*. Between studies, animals were kept in a main housing area. Quail were kept together in large cages and anoles were together in aquaria; and rats were kept in separate standard rodent cages. Each study was performed twice, once using

larval ticks and once using nymphal ticks. All experiments were approved by the Institutional Animal Care and Use Committee at Mississippi State University; IACUC protocol #08-039.

Host preference study

Using a paintbrush, immature ticks (n=50) were placed into the center of a PVC tube connecting two cages each with a different animal species. All three possible combinations of hosts (rat – anole, quail – rat, anole – quail) were used, with three replicates. Therefore, six animals of each species were used each time. At time zero, ticks were placed in the middle of PVC tubes and allowed to find a host, during which time cages were moved every six hours throughout the room to eliminate any possible airflow or temperature variables. After 24 hours, tubes were removed and any ticks left inside them were counted and eliminated from the study. Trays of water under each cage were then cleaned and searched daily for fallen engorged ticks. Engorged ticks were counted from each animal tray and taken back to the lab for placement in humidity chambers. Larval ticks were allowed to feed for seven days; nymphs for ten days. Unpaired *t*-tests were used for statistical analysis of the results.

Feeding success study

In a separate experiment, immature ticks (n=50 for larvae and n=40 for nymphs) were given no option of host and placed directly onto the animals' bodies, in the case of cotton rats and quail. In the case of the anoles, tick placement directly onto their bodies was attempted, but proved to be unsuccessful as ticks failed to stay on the skin of anoles. In this case, an open 0.5ml tube with the ticks was placed into each cage with an anole.

Ten individuals of each animal species were used. Cage setup was identical to the host preference study; however, PVC tubes were not used to connect cages. Each animal species was kept in a separate room. Again, trays of water were checked daily for fallen engorged ticks. Larvae were again allowed to feed for seven days; nymphs fed for ten days. All engorged ticks were taken back to the lab to be weighed and allowed to molt. Larvae from each individual animal were pooled and mean weights recorded. The nymphs, however, were individually weighed prior to placement in humidity chambers to await molting. Time to engorgement and percent molting success were recorded for both larval and nymphal ticks. Tick weights, days to engorgement, and molting success were analyzed using unpaired *t*-tests.

Results

Host preference study

Few engorged ticks were recovered from animal trays (17/450 larvae and 11/450 nymphs); most were found unfed in the trays of water. This may have reflected problems maintaining adequate humidity levels, which improved in later studies. However, although numbers were low, some trends were seen. Neither larval nor nymphal ticks fed on anoles. In addition, more ticks fed on quail (7 larvae, 10 nymphs) than on rats (4 larvae, 7 nymphs). When given a choice between with an anole and a quail, seven larvae and four nymphs fed on quail. When given a choice between a cotton rat and an anole, six larvae and two nymphs fed on rats. When a quail and a cotton rat were present, three larvae and three nymphs fed on quail and one larva and two nymphs fed on a rat. These results, however, were not statistically significant due to low numbers.

Feeding success study

Unfortunately, during the time of these experiments, one quail and one anole died due to causes unrelated to tick feeding; these animals were eliminated from the study. Results of the feeding success experiment are shown in Table 1. No larvae or nymphs fed on anoles. There were not enough engorged larvae to compare weights statistically. In addition, neither the percent molting success nor days to engorge was statistically different ($P>0.05$). The data for engorged nymphal weights for rats and quail appeared to be distributed normally according to qq plots. Using a pooled *t*-test, there was a strongly significant difference ($F=1.03$; df=115; $P<0.0001$) between weights of nymphs engorged on quail versus rats at the 5% significance level, with ticks engorging on rats being heavier. Nymphs engorged significantly sooner ($F=1.53$; df=117; $P<0.05$) on quail than on cotton rats. Percent molting success of nymphs was not statistically different among host species ($P>0.05$).

Discussion

Results from these studies suggest that Carolina anoles do not serve as hosts for *A. maculatum* larvae or nymphs in nature. This is in contrast to the natural history of other ixodid ticks, such as *I. scapularis*, which do feed on reptiles as immatures, both under laboratory conditions and in nature (Apperson *et al.*, 1993, Oliver *et al.*, 1993, James & Oliver, 1990). Our studies support previous reports that *A. maculatum* larvae and nymphs feed on bobwhite quail and cotton rats, and also suggest that reptiles do not play a role in this tick's life cycle. This has important implications for agents of disease harbored by *A. maculatum*, such as *R. parkeri*. In documenting the epidemiology of such pathogens, future studies should not focus efforts on reptiles, though other *Amblyomma*

species are found on reptiles in the southeastern United States (Nelder & Reeves, 2005, Corn *et al.*, 2011).

There were some noted differences between quail and cotton rats as hosts. While significant differences in host preference could not be determined in our studies, the characteristics of nymphal feeding on cotton rats compared to quail were statistically different. Our results support data from Koch and Hair (1975) regarding differences in feeding success of *A. maculatum* nymphs on various hosts. In that paper, proposed reasons for what may cause ticks to feed more successfully on certain host species included differences in quantity and quality of ingested bloodmeal between the hosts (Koch & Hair, 1975). While these factors may not directly affect pathogen acquisition and transmission and they were not addressed in that study, they may still influence the maintenance and transmission of a tick-borne pathogen indirectly.

In conclusion, our experiments offer information towards better understanding the life cycle of *A. maculatum* and, ultimately, the ecology and epidemiology of pathogens such as *R. parkeri*. Further studies are warranted to determine the extent to which *A. maculatum* vertebrate hosts, such as cotton rats and bobwhite quail, are involved as hosts of pathogens found in this tick.

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Table 3.1 Feeding success of larval and nymphal *Amblyomma maculatum* on bobwhite quail and cotton rats.

	quail (n=9)	rat (n=10)
Larvae (n=50)		
Total no. engorged (range per animal) avg # per animal (range)	22 (0-5) 2.4 (0-5)	43 (2-8) 4.3 (2-8)
Mean weight (range)	2.3 (2-3) mg	2.5 (1-5) mg
Mean days to engorge (range)	5.6 (5-6)	5.7 (5-6)
Percent molting success	86	77
Nymphs (n=40)		
Total no. engorged (range per animal) avg # per animal (range)	67 (0-17) 7.4 (0-17)	52 (0-13) 5.2 (0-13)
Mean weight (range)	13.2 (7-19) mg*	15.8 (7-21) mg*
Mean days to engorge (range)	7 (5-8)*	8.2 (6-9)*
Percent molting success	48	40

Results are not reported for anoles as no ticks fed on them.

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CHAPTER IV

EVIDENCE OF ANTIBODIES TO SPOTTED FEVER GROUP RICKETTSIAE IN SMALL MAMMALS AND QUAIL FROM MISSISSIPPI

Vector-Borne and Zoonotic Diseases, 12

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Abstract

Rickettsia parkeri is a recently recognized human pathogen primarily associated with the Gulf Coast tick, *Amblyomma maculatum*, with immature stages reported from wild vertebrates. To better understand the role of vertebrates in the natural history of this bacterium, we evaluated small mammals and ground-dwelling birds for evidence of infection with *R. parkeri* or exposure to the organism. We sampled small mammals

(n=39) and passerines (n=47) in both north-central and southeast Mississippi, while northern bobwhite (*Colinus virginianus*) samples (n=31) were obtained from farms in central Mississippi. Blood from all sampled animals was tested using PCR for spotted fever group rickettsiae (SFGR) and for antibodies to SFGR using *R. parkeri* antigen. Ectoparasite samples were removed from animals and included mites, lice, fleas, and immature ticks. Of 42 small mammal samples collected, seven were positive for antibodies to SFGR; none tested positive by PCR for DNA of SFGR. Of 47 passerine blood samples collected, none were positive for DNA of SFGR by PCR nor did any show serologic evidence of exposure. Finally, none of 31 northern bobwhite samples tested were positive for SFGR DNA, while seven were seropositive for rickettsial antibodies. Detection of seropositive rodents and quail suggests a role for these host species in the natural history of SFGR, possibly including *R. parkeri*, but the extent of their role has not yet been elucidated.

Keywords Gulf Coast tick, rickettsiae, birds, small mammals, ectoparasites

Introduction

Several tick-borne diseases have recently increased in incidence and geographic distribution, warranting classification as “emerging” infectious diseases (Gratz 1999, Fritz 2009). Rickettsiosis caused by *Rickettsia parkeri*, a member of the spotted fever group rickettsiae (SFGR), is a recently recognized tick-borne disease, first reported in a human in 2004 (Paddock et al. 2004). Although these diseases mainly affect humans, wildlife may play an important role in their persistence in nature. This role may include serving as a reservoir or amplifier of infection or as a primary host in the tick life cycle and as a dead-end for the pathogen.

Vertebrate hosts play a role in the natural maintenance of some rickettsial organisms. The causative agent of Rocky Mountain spotted fever (RMSF), *Rickettsia rickettsii*, has been reported to circulate at high enough levels in Colombian and golden-mantled ground squirrels, meadow mice, and snowshoe hares to infect naïve feeding ticks (Burgdorfer et al. 1966). In South America, capybaras and opossums (*Didelphis* spp.) have been implicated as amplifiers of this organism as well (Labruna 2009). *Rickettsia typhi* is maintained by a rat-flea life cycle (Azad 1990) and has also been found in spleen tissue samples from opossums (*Didelphis marsupialis*; Williams et al. 1992), suggesting that this opossum is a good host for proliferation of the bacterium. In Mississippi, mammals such as raccoons, opossums, cottontail rabbits, and white-tailed deer have been shown to have antibodies to SFGR (Norment et al. 1985, Castellaw et al. 2011). Identifying vertebrates that are part of tick-borne agent life cycles provides important information about the ecology and epidemiology of such pathogens relevant to veterinary and public health sectors.

Rickettsia parkeri is a member of the Alpha-proteobacteria, in the Family Rickettsiaceae, and is phylogenetically closest to *R. africae* (Fournier et al. 1998, Roux and Raoult 2000). This organism, like other SFGR, requires a vertebrate or invertebrate host for both proliferation and survival as it uses energy from its host cells (Weiss 1973). Because *R. parkeri* was only recently recognized as a pathogen after almost 70 years of being considered non-pathogenic (Paddock et al. 2004, Parker 1939), our understanding of its natural history is minimal. Although the main tick vector is *Amblyomma maculatum* (Parker 1939, Philip et al. 1978), the role played by vertebrate hosts of this tick in the cycle of *R. parkeri* is not known. Transovarial and transstadial forms of transmission are

important in perpetuating the life cycle for many rickettsiae (Azad and Beard 1998), thus immature stages of the ticks may be more important than adults in spreading infection to other ticks and naïve vertebrate hosts. Transovarially infected larvae have the potential to spread the infection to the vertebrate hosts they feed on, and transstadially to nymphal stages, which again have the ability to transmit the pathogen to their vertebrate hosts. Larval and nymphal Gulf Coast ticks are generally found on ground-dwelling animals (Bishopp and Hixson 1936, Hixson 1940), thus our objective was to evaluate wild birds and small mammals for exposure to and infection with SFGR such as *R. parkeri*. Ultimately, these data should contribute to an understanding of the natural history of SFGR in vertebrate hosts.

Materials and Methods

Small mammal samples

Sites in Mississippi for trapping small mammals were chosen based on the presence of *A. maculatum* as identified by previous studies (Goddard and Norment 1983, Goddard and Paddock 2005) or property owners. Trapping was performed at two locations (Figure 1) in winter 2009 and spring 2010, using Sherman live-traps. Traps were baited using peanut butter and oats and placed in a grid with 10 meters spacing between traps. Traps were checked starting at 0700-0800 the following morning and checked every two hours for the remainder of the day. Trapped small mammals were processed on site. Blood (0.3mL maximum) was collected from the saphenous vein using heparin-coated capillary tubes. The animal was then combed for ectoparasites as thoroughly as possible without causing undue stress, which were collected and placed into 70% ethanol. All mammals were then released at their original capture sites.

Avian samples

Passerines were caught in spring and summer 2009 using mist-nets in two north-central sites (Starkville: +33° 29’ 6.85”, -88° 46’ 41.13”; Mathiston: +33° 31’ 44.21”, -89° 7’ 54.97”) and at one coastal site (Moss Point: +30° 23’ 56.58”, -88° 27’ 28.99”) (Figure 1). Blood samples were collected from captured birds via jugular venipuncture (0.3mL maximum) with syringes interiorly coated with heparin. The birds’ heads were then examined for any attached ticks before being released at their capture sites. Sampling passerines was done with a Scientific Collection Permit from the Mississippi Department of Wildlife, Fisheries, and Parks (U.S. Fish and Wildlife Service permit MB027041-1).

We collected samples from two northern bobwhite farms in north-central Mississippi that housed quail in conditions amenable to ticks; specifically, birds were housed in runs on the ground with wire fencing. One farm was in Maben (+33° 34’ 37.64”, -89° 3’ 7.82”) and the other near Starkville (+33° 22’ 34.05”, -88° 41’ 30.98”). Blood was collected from the jugular vein as done for passerines. Quail were checked for ectoparasites around their heads then released.

DNA Extractions

Small mammal blood samples were extracted using GE Healthcare’s illustra blood genomicPrep Mini Spin kit (GE Healthcare, Piscataway, NJ). Avian blood was extracted using the QIAamp DNA Blood Midi kit (Qiagen Inc., Valencia, CA). In all cases, a 50µl volume of blood was extracted following manufacturer’s protocols.

Polymerase Chain Reaction (PCR)

A nested PCR assay targeting the rickettsial outer membrane protein A (*rompA*) gene specific for SFGR was used with primers 190-70 and 190-701 for the primary reaction and primers 190-FN1 and 190-RN1 for the secondary reaction (Sumner et al. 2007). *Rickettsia parkeri* DNA extracts (Tate's Hell strain) and non-template water controls were included in the nested PCR assay.

Indirect Fluorescent Antibody (IFA) test

Plasma from heparinized blood samples was tested to determine if SFGR-specific antibodies were present. Samples were screened at a 1:64 dilution using fluorescein isothiocyanate (FITC) anti-rat (KPL, Gaithersburg, MD) and FITC anti-mouse (KPL, Gaithersburg, MD) as secondary antibodies for rat samples and for other small mammal samples, respectively. FITC anti-bird (KPL, Gaithersburg, MD) was used for passerine samples and FITC anti-chicken (KPL, Gaithersburg, MD) was used for northern bobwhite samples. End-point titrations were determined on positive samples using two-fold serial dilutions ranging from 1:64 to 1:1024. Diluted samples were placed on *R. parkeri* antigen coated 12-well slides, incubated for 35min at 37°C, then washed in PBS followed by water. Appropriate FITCs were added to the wells and slides were again incubated at 37°C for 35min. Finally, slides were washed, counterstained with Eriochrome black T, and air-dried before applying VECTASHIELD® (Vector Laboratories, Inc., Burlingame, CA) and a coverslip.

Ectoparasite Identification

Samples were first sorted by Order (or Subclass Acari for mites) and then identified to species by specialists: Dr. Lance A. Durden at Georgia Southern University; Dr. Jerome Goddard at Mississippi State University; Dr. Hans Klompen at Ohio State University; and Dr. Richard Robbins of the Armed Forces Pest Management Board. Vouchers were deposited in the Mississippi Entomological Museum, Mississippi State University.

Results

Sample collection

Twenty-four samples were obtained from rodents trapped in Moss Point in winter 2009 (n=12) and spring 2010 (n=12) (Table 1). Small mammal samples obtained in Starkville were from animals trapped in association with a Mississippi State University course in winter 2009 (n=14) and independently in spring 2010 (n=1). Forty-seven passerines were caught by mist-net and sampled (May through July 2009) from Starkville (n=31), Moss Point (n=8), and Mathiston (n=8). Twenty northern bobwhite samples were obtained from the farm near Starkville (May 2010) and eleven were collected from the farm in Maben (November 2010).

PCR Results

No small mammal, passerine, or northern bobwhite samples tested positive for SFGR DNA by PCR. Positive controls showed bands of appropriate size on agarose gels.

IFA Results

Five rodent samples were seropositive. Two hispid cotton rats (*Sigmodon hispidus*) from Moss Point tested positive in winter 2009. Also at Moss Point, two cotton rats and one *Peromyscus* sp. tested positive in spring 2010. No passerines tested positive for SFGR antibodies; however, seven northern bobwhites from Starkville were seropositive. No northern bobwhites from the farm in Maben were seropositive. Endpoint titrations of positive samples are shown in Table 2.

Ectoparasite Samples

Four Orders of ectoparasites were found on rodents, including two species of ticks, three genera of mites, one sucking louse species, and two flea species (Table 3). All specimens were mounted for identification. No ectoparasites were found on birds or shrews.

Discussion

Cotton rats and northern bobwhite quail serve as important vertebrate hosts for immature stages of *A. maculatum* (Hixson 1937), and thus may play a role in the natural history of the *A. maculatum*-transmitted pathogen *R. parkeri*. However, our study provides evidence of exposure to SFGR as a group, which includes organisms such as *R. rickettsii* that uses *Dermacentor variabilis* as a primary vector. Therefore, these animals may have been exposed to different rickettsiae, particularly because both *D. variabilis* and *A. maculatum* were collected from rodents. On the other hand, a recent study reported 28% of unfed *A. maculatum* ticks from Florida and Mississippi to be infected with *R. parkeri*, representing a range of 11 to 40% in the individual counties sampled

(Paddock et al. 2010). This is in stark contrast to reported prevalence of generally less than 5% of *Dermacentor* ticks being infected with *R. rickettsii* (Burgdorfer et al. 1975, Linnemann et al. 1980, McDade and Newhouse 1986, Wikswo et al. 2008, Stromdahl et al. 2011).

While no rickettsial DNA was detected in blood samples, some animals showed strong antibody responses to *R. parkeri* antigen (specifically, two rodents from Moss Point had endpoints of 1:512). This suggests that these animals were exposed to SFGR likely sometime within the previous two months. Experimental infections using rabbits, guinea pigs, and mice suggested that those animals generally show a peak antibody response between 10 and 20 dpi. The authors noted that a high dose (5.6×10^6 plaque-forming units) was needed to elicit a response from the mice (Anacker et al. 1979). Samples from animals in the current study that showed higher titers for SFGR antibodies (rodents at Moss Point) were collected during peak activity times for both larval and nymphal *A. maculatum* – late fall and early spring (Hixson 1937, Hixson 1940).

Results from PCR tests did not reflect those of IFA assays, but rickettsial DNA can be present without being in circulation. Rickettsiae are obligate intracellular parasites infecting endothelial cells (Pinkerton 1942). These cells are in general not found circulating throughout the body, although they can enter the bloodstream as a result of damage to the endothelium (Silverman 1984, Valbuena and Walker 2009). Other rickettsiae have been detected in wildlife by PCR, but at very low rates. For example, *R. helvetica* DNA was identified in eight of 112 Sika deer samples in Japan (Inokuma et al. 2008). The PCR tests performed in this study did not detect rickettsial DNA in the blood; the organism may have been present in low numbers, or not circulating in the animals at

the time samples were taken. Experimental infections performed in our laboratory resulted in isolation of *R. parkeri* from cotton rat tissues including skin, blood, and spleen during acute infection but not chronic infection (Moraru et al., unpublished data). The current study did not incorporate euthanasia and necropsy of field animals, and so did not allow for collection of such samples, which would be important to test whether *R. parkeri* occurs in tissues other than blood. It would also be of interest in future studies to perform methodical checks for ectoparasites such that some could be tested for rickettsial DNA.

Cotton rats and northern bobwhite have shown evidence of exposure to SFGR and they are known hosts for *A. maculatum*, the primary vector of *R. parkeri* (Parker 1939, Sumner et al. 2007). If they do prove to play a role in the maintenance of *R. parkeri*, then this would have implications in the epidemiology of this pathogen. To gain a better understanding of the ecology of this system, future studies with these hosts should include serosurveys to detect *R. parkeri*-specific antibodies, testing of their ectoparasites for *R. parkeri*, and experimental infections with *R. parkeri*.

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Disclosure Statement

No competing financial interests exist.

Table 4.1 List of vertebrate species and number collected by capture site.

	Starkville	Moss Point	Mathiston	Maben
Small Mammals	Cotton Rat (4) Woodland Vole (1) House mouse (2) Southern Short-tailed Shrew (6) Eastern Woodrat (1) *Peromyscus* sp. (1)	Cotton Rat (22) *Peromyscus* sp. (2)	N/A	N/A
Passerines	Northern Cardinal (12) Northern Mockingbird (1) Carolina Wren (3) Brown Thrasher (1) Summer Tanager (1) Prothonotary Warbler (1) House Sparrow (5) Tufted Titmouse (1) Eastern Bluebird (2) Red-bellied Woodpecker (1) unknown (2) Blue Grosbeak (1)	Northern Cardinal (4) Northern Mockingbird (2) Carolina Wren (1) Gray Catbird (1)	Northern Cardinal (5) Eastern Wood Pewee (1) Carolina Wren (1) Brown Thrasher (1)	N/A
Quail	Northern Bobwhite (20)	N/A	N/A	Northern Bobwhite (11)

Table 4.2 Plasma titrations of rodents and bobwhite quail analyzed by immunofluorescent antibody test.

	No. positive (total sampled)	Titer Range
Sigmodon hispidus	4 (24)	1:64 – 1:512
Peromyscus sp.	1 (2)	1:512
Colinus virginianus	7 (20)	1:64 – 1:256

Rodents listed were trapped at Moss Point in Mississippi. All quail samples were obtained from a farm outside of Starkville in Mississippi.

Table 4.3 Ectoparasites found on rodents trapped in Moss Point, Mississippi.

	Number of Ectoparasites Collected (No. hosts)								
	Acari				Anoplura	Ixodida		Siphonaptera	
Host (Total No.)	*Androlaelaps* spp.	*Androlaelaps fahrenholtzi*	*Gigantolaelaps* spp.	*Ornithonyssus bacoti*	*Hoplopleura hirsuta*	*Amblyomma maculatum*	*Dermacentor variabilis*	*Orchopeas howardi*	*Polygenis gwyni*
December 2009									
Sigmodon hispidus (12)	4 (3)	0	0	0	4 (2)	0	0	1 (1)	0
Peromyscus (1)	0	0	6 (1)	0	0	0	1[a] (1)	0	0
May 2010									
Sigmodon hispidus (12)	19 (5)	0	0	2 (1)	2 (2)	2 (1)	2 (2)	0	5 (5)
Peromyscus (1)	0	2 (1)	0	0	0	0	0	0	0

Figure 4.1 Map of Mississippi showing vertebrate blood sampling sites for small mammals, passerines, and quail.

Circles show where both mammals and passerines were collected, diamonds represent locations of quail farms sampled, and the four-point star indicates where only passerines were examined.

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CHAPTER V

EXPERIMENTAL INFECTION OF COTTON RATS AND BOBWHITE QUAIL WITH *RICKETTSIA PARKERI*

Parasites & Vectors (in press)

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Abstract

Background

Amblyomma maculatum is the primary vector for *Rickettsia parkeri*, a spotted fever group rickettsia (SFGR) and human pathogen. Cotton rats and quail are known hosts for immature *A. maculatum*; however, the role of these hosts in the ecology of *R. parkeri* is unknown.

Methods

Cotton rats and quail were inoculated with low or high doses of *R. parkeri* (strain Portsmouth) grown in Vero cells to evaluate infection by *R. parkeri* in these two hosts species. Animals were euthanized 2, 4, 7, 10, and 14 days post-injection (dpi) and blood, skin, and spleen samples were collected to analyze by Vero cell culture and polymerase chain reaction (PCR). In a second trial, cotton rats and quail were inoculated with *R. parkeri* and nymphal *A. maculatum* ticks were allowed to feed on animals. Animals were euthanized on dpi 14, 20, 28, 31, and 38 and blood and tissues were collected for serology and PCR assays. Fed ticks were tested for *R. parkeri* by PCR and Vero cell culture.

Results

Rickettsia parkeri was isolated in cell culture and detected by PCR in skin, blood, and spleen tissues of cotton rats in the initial trial dpi 2, 4, and 7, but not in quail tissues. In the second trial, no ticks tested positive for *R. parkeri* by PCR or cell culture.

Conclusions

These studies demonstrate that viable *R. parkeri* rickettsiae can persist in the tissues of cotton rats for at least 7 days following subcutaneous inoculation of these bacteria; however, quail are apparently resistant to infection. *Rickettsia parkeri* was not detected in nymphal ticks that fed on *R. parkeri*-inoculated cotton rats or quail, suggesting an alternate route of transmission to naïve ticks.

Keywords: *Rickettsia parkeri*, experimental infection, cotton rat, quail

Background

Spotted fever group rickettsiae (SFGR) are vector-borne organisms often causing disease in humans. *Rickettsia rickettsii*, the causative agent of Rocky Mountain spotted fever (RMSF), is the best studied and most virulent of the SFGR [1]. The pathogenic potential of many other SFGR, however, is not well-documented, particularly for recently recognized rickettsial species. Further, while the genetic relatedness of known and emerging rickettsiae [2-4] and their presence in certain animal populations [5-7] have been described, basic ecology and epidemiology are less well-understood.

Despite the initial recognition of *R. parkeri* in 1937 [8], studies of this SFGR only increased substantially after 2004, when the first case of human infection was reported [9]. Subsequent seroprevalence surveys demonstrated certain animal species, including opossums, capybaras, and dogs, to be naturally exposed to *R. parkeri*, or a closely related SFGR [10-12].

Our understanding of the natural history of *R. parkeri* is mainly limited to its distribution in its primary tick vector, *Amblyomma maculatum*, commonly known as the Gulf Coast tick. *Rickettsia parkeri* has been detected in 12%-43% of questing adult Gulf Coast ticks collected across the southeastern United States [13-15], suggesting this *Rickettsia* species is efficiently transmitted from the nymphal stage to the adult stage. It is unknown, however, if immature *A. maculatum* acquire the microorganism predominantly by feeding from rickettsemic vertebrate hosts, through effective transovarial and transstadial transmission, or possibly both routes of transmission. Immature Gulf Coast ticks feed on small mammals such as cotton rats and ground-dwelling birds, including meadowlarks and northern bobwhite [16-18]. Adult stages parasitize larger mammals

including cattle, goats, deer, dogs, and occasionally humans [19]. Experimental infection studies showed that opossums (*Didelphis aurita*) and cattle seroconverted when inoculated with *R. parkeri*. Some animals (2/6 calves and 1/2 opossums) also became transiently rickettsemic [20, 21]. It is not known, however, if one or more vertebrate hosts act as reservoirs or amplifying hosts for *R. parkeri*, as described previously for *Rickettsia rickettsii*, the agent of Rocky Mountain spotted fever [22-24].

This study was performed to assess the infectivity of *R. parkeri* to cotton rats and bobwhite quail, two recognized vertebrate hosts for immature stages of *A. maculatum*, and to investigate the ability of immature ticks to acquire *R. parkeri* from these *R.* parkeri-exposed hosts.

Methods

Animal and tick sources

Cotton rats (*Sigmodon hispidus*) were purchased from Harlan Laboratories (Indianapolis, IN). Northern bobwhite quail were purchased from P & L Crowley Farm (Maben, MS).

Ticks were purchased from Texas A&M University (TAMU) and Oklahoma State University (OSU). Those from the latter institution have been previously found to be infected with "*Candidatus* Rickettsia andeanae", while ticks from the TAMU colony are not known to be positive for this organism (Moraru, unpublished data). Nymphal ticks obtained from both institutions were DNA extracted individually and PCR amplified using primers 16S+2 and 16S-1 to target the 16S rDNA gene as confirmation that tick DNA was extracted [25]. Extractions were then tested by PCR amplification targeting SFGR-wide *rompA* and "*Ca.* R. andeanae"-specific *rompA* gene fragments. The former

was done using primers 190-70 and 190-701 for the primary reaction and primers 190-FN1 and 190-RN1 for the secondary reaction [26]; the latter using primers Rx-190-F and Rx-190-R [14].

Culture for injections

Rickettsia parkeri was grown in Vero cell culture supplemented with minimum essential media (MEM) containing 10% fetal bovine serum. A low passage (P4 and P5) isolate of *R. parkeri* (Portsmouth) was used for all animal infections. Infected cultures were harvested when at least 90 percent of the Vero cells were infected, as determined by cell counts using 50µl in a hemocytometer.

Experimental exposure

The initial trial consisted of eleven quail and eleven cotton rats. All animals were pre-screened for SFGR antibodies via immunofluorescent antibody (IFA) testing (described in detail below). Five quail and five cotton rats received low dose injections of *R. parkeri* (1000 infected Vero cells in 0.2ml of culture media). Another set of five quail and five cotton rats were injected with a high dose of the organism (10 000 infected Vero cells in 0.2ml). Percent infectivity of Vero cells was estimated by cytospin, and cell counts were performed using a hemocytometer. Animals were injected subcutaneously, at the nape of the neck on cotton rats and in the right leg of quail. One individual of each species served as negative controls and were injected with 10 000 uninfected Vero cells in a 0.2ml volume. Four of twenty animals—one low dose quail, one low dose rat, one high dose quail, and one high dose rat—were numbered and randomly selected from each group for euthanasia at 2, 4, 7, 10, and 14 days post injection (dpi). The controls were

euthanized on dpi 14. Animals to be euthanized were numbered and selected at random, within their dose assignment, and euthanized using carbon dioxide.

Upon euthanasia, blood was collected from the animals via intracardiac puncture. A 250µl volume of whole blood from each animal was placed into individual flasks of confluent Vero cells. Skin from the injection site and spleen tissue samples were collected on necropsy. Half of each tissue sample was put into Vero cell culture (described in cell culture section below), and half was frozen at -20°C until DNA extractions and PCR assays could be performed.

Experimental tick infestation

The second trial consisted of eleven cotton rats and eleven quail. One cotton rat and one quail were injected with 10 000 uninfected Vero cells in 0.2ml of culture media. The remaining ten individuals of each species received injections of *R. parkeri* infected Vero cell culture (10 000 cells in 0.2ml each). On dpi 4, nymphal *A. maculatum* ticks were placed on each of the animals. Two cotton rats and two quail had OSU ticks (n=50), while all remaining animals (including controls) received TAMU ticks (n=65). Trays underneath each animal's cage were examined daily for fallen engorged ticks. All fallen ticks were placed in humidity chambers (90% RH) and allowed to molt. Ticks were allowed to feed for 13 days, after which a blood sample was taken from the animals for IFA testing (dpi 17).

Animals were euthanized on dpi 20, 24, 31, and 38. Upon euthanasia, a blood sample was collected via intracardiac puncture. At necropsy, tissues including skin from the original injection site, liver, spleen, kidney, and scrotal tissue (male rats only) were collected and stored at -20°C until DNA extraction and PCR testing could be performed.

All experiments were approved by the Institutional Animal Care and Use Committee at Mississippi State University (IACUC 10-067).

Indirect Fluorescent Antibody (IFA) test

Plasma from the blood samples was used to determine if SFGR IgG antibodies were present. Samples were screened at a 1:64 dilution. A 1:60 dilution of fluorescein isothiocyanate (FITC) anti-rat IgG (H+L) (KPL, Gaithersburg, MD) was used as a secondary antibody for rat samples; FITC anti-chicken (H+L) (KPL, Gaithersburg, MD) was used for the quail at a dilution of 1:275. Cotton rats and bobwhite quail known from previous IFA screening studies to be seronegative or seropositive for SFGR were used as controls.

Cell culture of vertebrate tissues and ticks

Tissues, of approximately $1cm^2$, from animals in the first trial were triturated, using a sterile scalpel blade, into 250µl of MEM + 10% FBS and added to $25cm^2$ flasks of Vero cell culture. All flasks (3 different tissues per individual animal) received 10µl penicillin-streptomycin (10000U/ml penicillin and 10mg/ml streptomycin).

In the second trial, ticks that successfully molted after feeding on *R. parkeri*-inoculated animals were pooled from each individual host. For example, all ticks that fed on quail 1 were put into one culture flask. Ticks were prepared for culture following a previously described protocol [27]. Briefly, they were put through a series of disinfecting washes. Each pool of ticks was placed into a 15ml tube with 10ml of a wash solution. For each wash, the tubes were vortexed for 3min, after which the liquid was aspirated out. Washes were, in order: hydrogen peroxide, 70% ethanol, 20% household bleach, and

sterile PBS. After this series of washes, ticks were cut using a sterile scalpel blade in a sterile petri dish, one at a time. Each tick was placed onto 0.2ml cell culture media and bisected longitudinally. One half was retained and placed at -20°C for subsequent DNA extraction and PCR testing. The other half was triturated in the media in the petri dish and then placed in a 25cm^2 culture flask along with any other triturated ticks that had fed on the same animal. Each flask also contained 100µl of penicillin-streptomycin (10000U/ml penicillin and 10mg/ml streptomycin) and 5µl of amphotericin (250µg/ml).

Two days after tissues and ticks were placed in culture, flasks were emptied and fresh media was added. Flasks were then monitored weekly for three to six weeks for infection using cytospin preparations and acridine orange staining. Briefly, slides were allowed to air-dry and then were placed in methanol for 10min. Slides were then flooded with acridine orange for 2-3min.

DNA Extractions

Rodent blood samples were extracted using GE Healthcare's illustra blood genomicPrep Mini Spin kit (GE Healthcare, Piscataway, NJ). Quail blood was extracted using the QIAamp DNA Blood Midi kit (Qiagen Inc., Valencia, CA). In all cases, 50µl of blood was extracted following the kit protocols as supplied by the manufacturer was followed.

Cell culture and ticks halves were DNA extracted using GE Healthcare's illustra tissue and cells genomicPrep Mini Spin kit and following the manufacturer's protocols (GE Healthcare, Piscataway, NJ). A 200µl volume was extracted from all harvested cultures. Tick halves were triturated into extraction buffer using a new sterile scalpel blade for each individual sample. Final elution volumes were 200µl.

Polymerase Chain Reaction (PCR)

A nested PCR program targeting a segment of the rickettsial outer membrane protein A (*rompA*) gene was used with primers 190-70 and 190-701 for the primary reaction and primers 190-FN1 and 190-RN1 for the secondary reaction [26]. *Rickettsia parkeri* DNA extracts (Portsmouth) and non-template water controls were included in the assays.

To test tick samples, this *rompA* PCR was preceded by a reaction using primers 16S+2 and 16S-1 to target the 16S rDNA gene as confirmation that tick DNA was extracted [25]. Ticks were tested with primers Rx-190-F and Rx-190-R, specific for "*Ca.* R. andeanae" and using a single reaction PCR assay [14].

Results

***Rickettsia parkeri* experimental infection in vertebrates**

The pre-inoculation serum samples obtained from all cotton rats and quail revealed no evidence of antibodies reactive with SFGR at a dilution of 1:64 or higher. Cotton rats and quail euthanized on dpi 2 and 4 were not seropositive; however, animals euthanized on dpi 7, 10, and 14 were seropositive. Control animals were seronegative. No blood samples tested positive by PCR at the time of euthanasia. No tissues tested directly by PCR were *rompA* positive.

Results of PCR assays are shown in Table 1. Briefly, rickettsial DNA was detected in the skin sample of a cotton rat at dpi 4, and also in cultures of blood, skin, and spleen from cotton rats. *Rickettsia parkeri* was re-isolated from blood, skin, and spleen tissues from cotton rats, but not from any quail tissues.

Experimental infections with ticks

In the series of experiments where *R. parkeri*-infected animals were exposed to nymphal *A. maculatum*, 2 *R. parkeri*-exposed rats died, due to undetermined causes before completion of the tick feeding period (one on dpi 13 and one on dpi 15). Time of death allowed for blood to be obtained from only one of these rats. The control rat died on dpi 24, also from unknown causes apparently unrelated to the study; blood and spleen samples were collected and tested by PCR and found to be negative for SFGR DNA.

All animals were seronegative on pre-screen by IFA. Controls remained seronegative throughout the study. On dpi 17, 8 of 9 rats and 5 of 10 quail were positive for SFGR antibodies at a 1:64 dilution. No blood sample at this time-point tested positive by PCR. Two quail (one sampled dpi 31 and the other on dpi 38) were seropositive (1:64) at time of euthanasia. All rats were seropositive (1:64) at time of euthanasia. No blood samples or other animal tissues tested positive by PCR at any time point.

A total of 61 engorged ticks were recovered from the quail (0-12) and cotton rats (0-8), representing a range of 0-7 ticks per animal and resulting in 13 culture flasks (1-7 ticks per animal). All cell cultures and ticks tested negative by PCR for rickettsial DNA. OSU ticks remained positive for "*Ca.* R. andeanae" after feeding on animals.

Discussion

This study demonstrates that needle-inoculated cotton rats can maintain infection with *R. parkeri* in various tissues for at least 4-7 days. Nonetheless, *R. parkeri* appears to be rapidly cleared by the immune system of cotton rats and even more quickly in bobwhite quail. Previous work with cotton rats indicated that *R. rickettsii* was cleared within 24 hours of infection [23].

Although some rickettsiae have vertebrate reservoirs or amplifiers [28], in other cases, the tick vector is implicated as a reservoir of certain rickettsiae. *Rickettsia honei* occurred in 63% of *Aponomma hydrosauri* ticks collected (n=46), but in no lizard hosts (n=17) [29]. Infection rates of *A. maculatum* with *R. parkeri* are also high. In Virginia, rates of infection with *R. parkeri* greater than 40% in *A. maculatum* have been reported [15, 30]. One study reports this rickettsia in 28% of *A. maculatum* ticks sampled in Mississippi and Florida, with a maximum infection rate of 40% in Jackson County, Mississippi [13]. Another study found a prevalence of 15.2% in *A. maculatum* collected throughout Mississippi [31]. Additionally, wild-caught rodents and bobwhite quail from farms in Mississippi have shown serological evidence of exposure to SFGR (Moraru et al., *in press*).

It appears that the ecology of *R. parkeri* is not dependent upon cotton rats or quail as reservoirs. While some feeding ticks may acquire the SFGR by feeding on recently infected animals such as cotton rats, there may be another mode of horizontal transmission occurring. Although our samples were small, our results suggest cotton rats and quail do not effectively transmit *R. parkeri* to naïve ticks. However, we also performed needle inoculations, which may result in different infection dynamics than feeding infected ticks on naïve animals. Transmission via co-feeding, from an infected tick to a naïve tick feeding nearby on the host, has been demonstrated with *R. massilae* and *R. conorii* [32, 33], suggesting the tick vectors may be acting as reservoirs. This would mean a less important role for vertebrate hosts in terms of pathogen maintenance. This merits further investigation in the context of *R. parkeri*.

Conclusions

This study adds valuable information to our limited knowledge of the dynamics of *R. parkeri* in avian and mammalian hosts. Cotton rats may serve briefly as sources of infection for feeding ticks, and there may be other vertebrate species that also have this potential. While did not show direct evidence of infection with *R. parkeri*, they may still have the potential to infect naïve *A. maculatum* ticks. With the increasing number of recognized *R. parkeri* cases in humans, it is important to identify potential sources of infection. Many questions remain and the ecology of this tick-borne rickettsia still needs to be examined thoroughly, both with surveys of wild animals and under experimental settings.

Competing Interests

The authors declare that they have no competing interests

Authors' contributions

GMM contributed to study design, laboratory work, intellectual interpretation, and writing of the manuscript. JG was involved in study design, intellectual interpretation, and revision of the manuscript. CDP contributed valuable intellectual content and revised the manuscript critically. AVS supervised and designed the study, contributed intellectual content, and performed critical revisions of the manuscript.

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Table 5.1 PCR results from experimental infection with *R. parkeri* in cotton rats via injection.

	dpi		dpi 4		dpi 7		dpi 10		dpi 14	
	Low	High	Low	High	Low	High	Low	High	Low	High
Skin	- / +*	- / +	- / +	+ / +	- / +	- / -	- / -	- / -	- / -	- / -
Blood	- / -	- / -	- / +	- / +	- / -	- / -	- / -	- / -	- / -	- / -
Spleen	- / -	- / -	- / -	- / +	- / -	- / -	- / -	- / -	- / -	- / -

Tissues were PCR tested and placed in Vero cell culture. "Low" and "high" indicate the dose the animal received (10000 or 100000 infected *R. parkeri* Vero cells). Days post injection (DPI) across the table represent time of euthanasia and tissue collection.
* Signs before the slash indicate tissue results, and signs after the slash signify results of cultures.

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CHAPTER VI

UTILITY OF REVERSE LINE BLOT HYBRIDIZATION FOR BLOODMEAL ANALYSIS IN ARCHIVED *AMBLYOMMA MACULATUM*

Abstract

The Gulf Coast tick, *Amblyomma maculatum*, is a significant pest of livestock and a vector for human and canine pathogens. Of primary importance is the role of *A. maculatum* in transmission of *Rickettsia parkeri*, a recently recognized human pathogen causing spotted fever rickettsiosis in the Western hemisphere. In this study, we investigated the utility of reverse line blot hybridization (RLBH) for identifying previous hosts fed on by adult *A. maculatum* in order to further evaluate the role of these hosts in the natural history of *R. parkeri.* Genomic DNA extracts from individual adult *A. maculatum* that were collected using drag cloth from sites in Mississippi during 2008-2010 were used in a polymerase chain reaction (PCR) to amplify a region of the mitochondrial 12S rRNA gene. PCR products were subjected to RLBH using oligonucleotide probes specific for Classes Mammalia, Aves, and Reptilia, Orders Rodentia and Artiodactyla, and the genus *Homo*. We detected human DNA and cross-contamination in our samples, similar to what has been previously reported for this technique. As primers for the gene of interest amplify from a broad range of vertebrates, and human DNA contamination is not uncommon, precautions should be taken during

initial DNA extraction. This manuscript discusses various approaches taken to optimize the use of RLBH for our samples.

Keywords: bloodmeal detection, reverse line blot, *Amblyomma maculatum*, 12S rDNA

Introduction

Tick-borne pathogens are of medical and veterinary importance worldwide (Jongejan & Uilenberg, 2004). Identifying potential vertebrate reservoirs or amplifiers of tick-borne pathogens often requires capturing wild hosts, which can prove difficult, hazardous, inefficient and costly. Molecular techniques now exist that allow for identification of traces of vertebrate host DNA in field-collected ticks and circumvent the initial need for handling wild hosts.

Reverse line blot hybridization (RLBH) was first developed to type group A streptococci using *emm* gene polymorphisms (Kaufhold *et al.*, 1994). The technique has since been adopted for various applications in vector-borne diseases, including identification of vertebrate hosts of blood-feeding arthropods (Kirstein & Gray, 1996). Various genes have been targeted for this purpose, such as the 12SrRNA gene (Humair *et al.*, 2007, Cadenas *et al.*, 2007), the 18S rRNA gene (Pichon *et al.*, 2003, Pichon *et al.*, 2005), and the *cytochrome b* gene (Kirstein & Gray, 1996). Molecular techniques such as PCR-restriction fragment length polymorphism (RFLP) and heteroduplex analysis have also been used to detect vertebrate host DNA (Kent, 2009). RLBH, however, is less expensive and somewhat less complicated in mastering (Kent, 2009).

The ecology of the Gulf Coast tick, *Amblyomma maculatum* (Sumner *et al.*, 2007, Parker, 1939), has been only generally described (Hixson, 1940, Teel *et al.*, 2010,

Bishopp & Hixson, 1936, Hixson, 1937). For example, immature stages feed on ground-dwelling birds such as meadowlarks and bobwhite quail and on small mammals including cotton rats (Hixson, 1940, Ellis, 1955, Kellogg & Calpin, 1971, Peters, 1936, Barker *et al.*, 2004). Identifying primary vertebrate hosts used by immature *A. maculatum* would prove useful for further investigating what vertebrate species might serve as amplifiers or reservoirs of the human pathogen it transmits, *Rickettsia parkeri*. Gulf Coast ticks have a geographic range spanning the Gulf and Atlantic coasts inward, with Mississippi in the center of the range along the Gulf Coast, and a state where human disease with *R. pakeri* has been reported (Paddock *et al.*, 2008, Finley *et al.*, 2006).

Rickettsia parkeri was first identified in 1939 (Parker, 1939), although it was only in 2004 that it was reported to cause human disease(Paddock *et al.*, 2004). Its only recent association with human disease left *R. parkeri* largely unstudied for over six decades. Like other members of the spotted fever group of rickettsia, it likely undergoes transovarial transmission, relying on ticks as a primary reservoir, and possibly vertebrate hosts as additional reservoirs or as amplifiers in nature. As vertebrate reservoir hosts have been described for other rickettsiae (Labruna, 2009), identifying the primary vertebrate species used by *A. maculatum* would be important for future studies investigating the role of these vertebrates in directly supporting the natural cycle of *R. parkeri.* This study was performed to determine host diversity of nymphal *A. maculatum* in Mississippi by analyzing bloodmeal remnants in unfed adult *A. maculatum*.

Methods

Sample collection

Unfed adult *A. maculatum* (n=707) were collected as part of a previous study using drag sampling from ten sites throughout the state of Mississippi between 2008 and 2010 (Ferrari *et al.*, 2012). Ticks collected during that study were stored in 70% ethanol until DNA extraction. Of these ticks, 698 had amplifiabletick 16S rDNA as per a pervious protocol (Black & Piesman, 1994), and were available for further testing. Blood and tick samples used for positive controls originated from previous experimental studies in our laboratory (Moraru et al., unpublished data). These included partially engorged adult female *A. americanum* fed on white-tailed deer (*Odocoileus virginianus*), adult *A. maculatum* fed on cotton rats (*Sigmodon hispidus*) and bobwhite quail (*Colinus virginianus*) as nymphs, and whole blood from these animals.

DNA extraction

Genomic DNA was extracted from whole ticks using the illustra tissue and cells genomicPrep kit (GE Healthcare) as part of a previous study (Ferrari *et al.*, 2012). Blood samples used as positive controls were extracted using either the illustra blood genomicPrep Mini Spin kit (GE Healthcare, Piscataway, NJ) or the QIAamp DNA Blood Midi kit (Qiagen Inc., Valencia, CA).

Polymerase chain reaction (PCR)

For detection of vertebrate DNA, extracts were first subjected to a touchdown PCR targeting a ~145bp fragment from the 12S rRNA gene, based on protocol by Humair et al. (2007). The reaction was carried out in 50µl volumes, using 1.5mM $MgCl_2$,

0.2mM dNTPs, 0.8µM of each primer, and 1.25 U *Taq*DNA polymerase (Promega, Madison, WI). In later trials, a glycerol-free *Taq*DNA polymerase (B-Bridge International Inc., Cupertino, CA) was used. Ten microliters of DNA extraction samples were used for each reaction. Briefly, the reaction began with a 3 min denaturation step held at 94°C, followed by one cycle of 94°C for 20 s, 60°C for 30 s, and 72°C for 30 s. The touchdown cycles began with 20 s at 94°C, followed by an annealing step of 30 s at 67°C and decreasing 1°C per cycle until this reached 52°C, and finally 30 s at 72°C. This was followed by 25 cycles of 94°C for 20 s, 52°C for 30 s, and 72°C for 30 s. The final extension step was 7 min at 72°C. PCR products were run on a 2% agarose gel stained with ethidium bromide and visualized under ultraviolet light to confirm amplification. Products were then stored at -20°C until use in RLBH. DNA extracts of blood samples from white-tailed deer or wild boar (*Sus scrofa*) were used as positive controls in PCR assays for the 12S rRNA gene. Non-template water controls were included in PCR assays amplifying fragments of both the 12S rRNA gene and tick mitochondrial 16S rRNA gene.

Reverse line blot hybridization (RLBH)

We chose previously published oligonucleotide probes for bloodmeal analysis by RLBH (Humair et al., 2007). The RLBH protocol was adapted based on previous studies, primarily by Humair et al., as well as other groups (O'Sullivan *et al.*, 2011, Scott *et al.*, 2012, Humair *et al.*, 2007). Probes were diluted from 2pM to 500pM to determine optimal concentrations and were bound to a biodyne-C membrane activated with 16% w/v 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC). The membrane was briefly rinsed with nanopure water and then placed in a MiniSlot^{TM} apparatus

(Immunetics Inc., Boston, MA) on top of 3 wet filter papers. Here, a 150µl volume of each diluted oligonucleotide probes was loaded (one into each lane) and incubated for 1min before being aspirated off of the membrane. Any empty lanes were marked with diluted pen ink. The membrane was then placed in 100mM NaOH for 8 min gently on a shaker, followed by an incubation step in 2xSSPE/0.1%SDS for 5 min at 60°C. Afterwards, we transferred the membrane to 20mM EDTA (pH 8) for 20 min and stored it in a Ziploc bag at 4°C until use, or immediately proceeded to hybridization with the sample PCR products.

PCR products were diluted in 2xSSPE/0.1%SDS at dilutions ranging from 1:10 to 1:35, similar to other RLB protocols (Rijpkema *et al.*, 1995, Gubbels *et al.*, 1999, Abbasi *et al.*, 2009). They were then heated to 100°C for 10 min, followed immediately by placement on ice. The membrane was transferred to 2xSSPE/0.1%SDS for 5 min (this was also attempted in buffer warmed to 52°C, 60°C, or 62°C) in preparation for hybridization. An additional blocking step was also attempted prior to this step, using 0.5% casein for 3 min. The membrane was then put into a MiniBlotter^{TM} 20 (Immunetics Inc., Boston, MA), on top of a foam cushion. Lanes were loaded either with 350µl of diluted PCR product or 350µl 2xSSPE/0.1%SDS. The apparatus was incubated for one hour at various experimental temperatures of 42°C, 52°C, or 60°C, 62°C, and 70°C. When incubated at 52°C, this step was preceded by an additional incubation of 30 min at 75°C. After hybridization, PCR products were aspirated from the lanes of the Miniblotter^{TM}. The membrane was removed, washed twice in 2xSSPE/0.1%SDS or 2xSSPE/0.5%SDS for 10 min at 52°C or 62°C, and then incubated for 45 min at 42ºC in streptavidin-peroxidase diluted in 2xSSPE/0.5%SDS (O'Sullivan *et al.*, 2011).

Sequencing of PCR products

To address cross-reactions, PCR products generated by amplification of the 12S rRNA gene fragment in DNA extracted from two separate ticks known to have fed on a quail and cotton rat, were purified and bi-directionally sequenced (Eurofins Operon, Huntsville, AL) to confirm identity.

Results

By modifying previous approaches, we developed a suitable RLBH protocol for testing archived *A.maculatum* ticks. The mammal, rodent, artiodactyl, and human oligonucleotides showed least cross-reaction when 200pmol were put onto the membrane; the bird and lizard probes were used at 100pmol. PCR products were finalized to be used at 1:35 dilutions and loaded onto a membrane prepared in 0.5% casein and 62°C 2xSSPE/0.1%SDS. The optimal hybridization temperature was determined to be 70°C, and the first wash was in 2xSSPE/0.5%SDS at 62°C.

The main issue we experienced with the protocol was cross-reactivity of PCR products with various oligonucleotide probes. Cross-reactions with the artiodactyl probe (Figure 1) were resolved by switching to a glycerol-free *Taq*DNA polymerase (B-Bridge International Inc., Cupertino, CA). Several times, DNA extractions of avian blood hybridized with both avian and mammalian probes (Figure 2). Unfortunately, we did not obtain a suitable nucleotide sequence for analysis from the DNA sample of the tick with a previous cotton rat bloodmeal; however, the nucleotide sequence generated from the DNA sample of the tick with a previous quail bloodmeal was, surprisingly, 100% identical to *Sigmodon hispidus* 12S rRNA gene sequences available in BLAST (Basic Alignment Search Tool; National Center for Biotechnology Information).

We approached subsequent RLBH assays using PCR amplifications generated with new aliquots of the touchdown PCR primers, non-template water controls and one positive control (DNA extraction from a wild boar blood sample). This was successful, with only the positive sample reacting only with the mammal and artiodactyl probes.

Once contamination issues appeared resolved and RLBH blots appeared to lack evidence of cross-reacting probes, field-collected adult *A. maculatum* were tested. The ticks and the extraction water hybridized only with the mammal probe, suggesting continued human DNA contamination. The positive control (wild boar) continued to react as expected, and no additional cross-reactions were observed. Subsequently, an oligonucleotide probe for *Homo sapiens* DNA was ordered and RLBH was repeated with the same samples, showing that these samples in fact had contamination with human 12S rRNA products. Additional RLBH attempts also revealed that many samples, including water controls, reacted with the human probe.

A difference was noted when using two different hybridization temperatures for the same PCR products. Avian blood samples and a DNA extraction of a tick reacted only with the avian probe when held at a hybridization temperature of 70°C, whereas at 62°C they also reacted with the mammalian probe (Figure 3). This was repeated using field-collected ticks; while those samples continued to react with the human oligonucleotide, they did also react with other probes, indicating true "hits".

Discussion

Initial PCR assays failed to produce product as they likely contained an excess of DNA template; we therefore optimized the PCR reaction by lowering the amount of template to the final volume described above. This resolution allowed our efforts to be

focused on the RLBH. The avian oligonucleotide probe, although shown to be specific by Humair et al. (2007), was not specific in our hands. This was likely due to contamination of many of our avian DNA extracts (during extraction) with human DNA, a contaminant that has been reported in other studies (Scott *et al.*, 2012, Humair *et al.*, 2007). In fact, Humair et al. (2007) eventually did not include the human or mammalian probes in their analyses of field-collected ticks, as they also experienced human DNA contamination. Non-specific binding may have occurred if hybridization temperatures were low enough to allow for non-specific annealing with mammalian probes (M.C. Scott, personal communication).

The sequence data demonstrating cotton rat DNA in a tick which had fed on a quail suggests contamination between samples while performing either DNA extractions or PCR setup. Thus, future RLBH assays should be performed using additional measures to prevent cross-contamination, as the method appears to be highly sensitive to other DNA that may be present in extractions.

Previous studies analyzing traces of bloodmeal have had variable success. Identification of host DNA was achieved in 43.6% (578/1326) of *Ixodes ricinus* ticks collected in Switzerland and targeting the 12S rRNA mitochondrial gene (Cadenas *et al.*, 2007). Humair et al. (2007) showed similar results with *I. ricinus*, reporting detection of host DNA in 48.6% (53/109) of ticks analyzed. Vertebrate DNA was identifiable in 62.8% (869/1383) of *A. americanum* nymphs in one study targeting a fragment of the 18S rRNA gene (Allan *et al.*, 2010) and in 47.17% (409/869) in another study using the 12S rRNA as a target (Goessling *et al.*, 2012). An optimized protocol for RLBH performed with ticks collected in the North America showed a 53.98% success rate (Scott *et al.*,

2012). These low rates may be explained by degradation of host DNA in the tick samples; DNA may degrade with longer periods of time between molt and DNA detection. This is a question worth investigating, specifically with tick samples. Additionally, while host DNA may come from skin cells ingested by feeding ticks as a result of attachment, erythrocytes are not nucleated in mammals, possibly adding to detection difficulties.

Various studies report detection of more than one previous host (Allan *et al.*, 2010, Scott *et al.*, 2012, Humair *et al.*, 2007). Allan et al. (2010) report 16.2% (141/869) of nymphal *A. americanum* ticks as hybridizing with more than one taxonomic probe. Ticks experiencing interrupted feeding may be occurring more than was previously thought, or some other ecological process may be taking place. Scott et al. (2012) also propose that DNA from the host of the larval stage may be detected along with that of the host used by the nymphal stage. Cross-reactions, however, are not readily interpreted.

In the future, RLBH might benefit by focusing on other gene targets for vertebrate hosts. For example, the *cytochrome c oxidase I* (COI) gene exhibits a faster rate of evolution than both the 12S rDNA gene, therefore suggesting that COI might be more useful in distinguishing among different vertebrate hosts (Kent, 2009). It would not, however, resolve human DNA contamination. This is something that would need to be addressed in the care taken when performing DNA extractions.

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Figure 6.1 Reverse line blot assay showing cross-reactions of host DNA in *Amblyomma maculatum* ticks.

Shown are cross-reactions with rodent and artiodactyls oligonucleotide probes. This was later resolved by using glycerol-free *Taq*DNA polymerase. Samples included were a negative water control (W), adult ticks fed on deer (M1, M2, F1, F2), a field-collected adult female (F12), a pool of 10 nymphs (nym) fed on rabbits, and blood from a white-tailed deer (628).

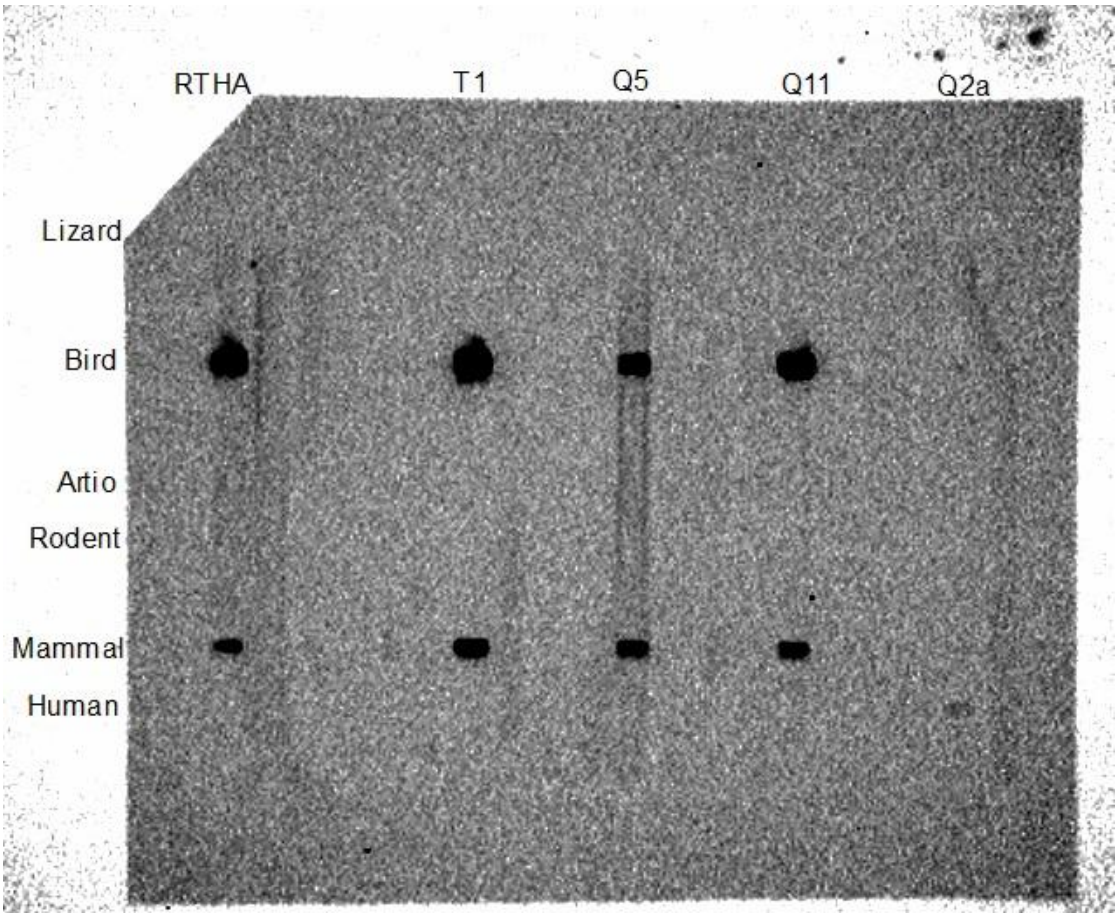

Figure 6.2 Reverse line blot assay of avian tissue samples to attempt elimination of cross-reaction with mammalian oligonucleotide probes.

Samples shown include DNA extractions of blood from a Red-tailed hawk (RTHA), blood from a turkey (T1), blood from a bobwhite quail (Q5), liver of a bobwhite quail (Q11), and half of a tick that was fed on a bobwhite quail as a nymph (Q2a). Unmarked lanes between samples contained either negative water controls or hybridization buffer (2xSSPE/0.1%SDS). Of note is cross-reactivity of samples with bird and mammalian oligonucleotide probes. Additionally, presence of human DNA can be seen in the Q2a sample.

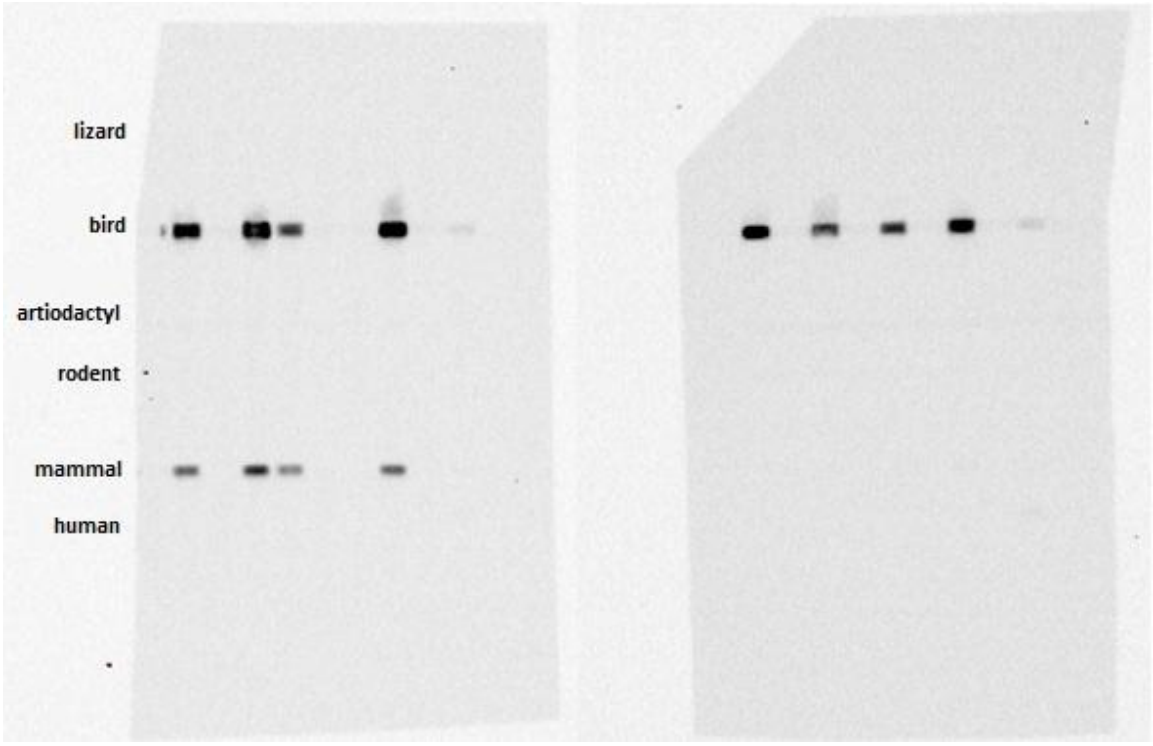

Figure 6.3 Reverse line blot assays performed under same conditions excepting hybridization temperatures and wash stringency post-hybridization.

The hybridization step was done at 62°C for the blot on the left, and at 70°C for the blot on the right. Oligonucleotide probes are noted alongside the membranes, and were placed on both in the same order. Samples were blood samples from birds (quail, turkey) and one tick that was fed on a quail (last lane, faint bands).

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CHAPTER VII

OVERALL DISCUSSION AND CONCLUSIONS

The studies presented here contribute to our knowledge of the ecology of the rickettsial agent *R. parkeri* and its primary tick vector, *A. maculatum*. The experimental and field work described herein have added valuable information to the subject. Overall conclusions and implications:

1. *Amblyomma maculatum* larvae and nymphs did not feed on anole lizards (*Anolis carolinensis*), suggesting these vertebrates are not important in the life cycle of the Gulf Coast tick, and therefore are not involved in the ecology of *R. parkeri*.
2. Nymphal *A. maculatum* fed significantly longer on cotton rats (*Sigmodon hispidus*) than on quail (*Colinus virginianus*), which could allow for better transmission of microorganisms to cotton rats compared with quail, suggesting that cotton rats are more important to pursue than quail in *R. parkeri*-centered studies.
3. Engorged nymphal *A. maculatum* which fed on cotton rats were significantly heavier than those fed on quail, which may influence acquisition of pathogens from the host and timing and completion of the tick life cycle. This could have implications for the ecology of tick-borne diseases such as *R. parkeri*.

4. Wild rodents in Mississippi showed evidence of exposure to spotted fever group rickettsiae, which could be a result of exposure to *R. parkeri* or any number of SFG organisms. This would signify that ticks carrying rickettsiae fed on these animals.
5. Farm-raised quail in Mississippi showed evidence of exposure to spotted fever group rickettsiae, which could include *R. parkeri*. This again would suggest that ticks infected with SFG rickettsiae fed on these quail.
6. Rickettsiae were isolated from cotton rats experimentally inoculated with *R. parkeri* from skin, blood, and spleen tissue samples taken during the acute phase of infection. This suggests that the organism travels through these organs in cotton rats and may replicate in one or several of them. Cotton rats could therefore harbor *R. parkeri*.
7. Experimentally, quail and cotton rats did not show evidence of rickettsemia when inoculated with *R. parkeri*. This may have been due to dose administered or it may imply that cotton rats and quail are able to quickly clear the organism from circulation, indicating that while they could be amplifiers or reservoirs of *R. parkeri*, a bloodmeal from these hosts would not serve as a good source of *R. parkeri* for naïve feeding ticks.
8. Nymphal *A. maculatum* fed on experimentally inoculated cotton rats and quail did not acquire infection with *R. parkeri*. This may have been due to the animals not receiving high enough doses of *R. parkeri* or to the ticks being placed on the animals too early or late in the infection.

Alternatively, it may be that a bloodmeal from these animals is not a good source of *R. parkeri* for naïve feeding ticks and that ticks acquire *R. parkeri* via co-feeding mechanism.

9. A reverse line blot protocol was developed for the purpose of identifying sources of bloodmeal for field-collected *A. maculatum*. This would prove useful in selecting target species for disease studies.

These conclusions and implications should help to guide future research in this area. The results suggest that the animals examined (rodents, passerines, bobwhite quail, and anoles) are not acting as reservoirs for *R. parkeri*. Importantly, it should be noted that this work represents single attempts at field work and experimental infections; these animals are still worth exploring as possible reservoirs. Perhaps bobwhite quail and cotton rats may also be important as a medium for transmission of the pathogen between ticks (co-feeding). An infected tick feeding in the area of the same host may infect a neighboring naïve tick. The vertebrate then allows for this exchange, and it is the tick instead that is the primary reservoir for *R. parkeri*.

On the other hand, it could be that another vertebrate that has yet to be examined is in fact a reservoir or an amplifier of *R. parkeri*. One possibility is the eastern meadowlark (*Sturnella magna*), which has been shown in other studies to be commonly parasitized by immature stages of *A. maculatum*.

This work has therefore answered some questions, but it has also opened the door for future research into questions concerning the ecology of the emerging pathogen, *R. parkeri*.

APPENDIX A

ADDITIONAL INFORMATION FOR CHAPTER III

Host preference cage setup

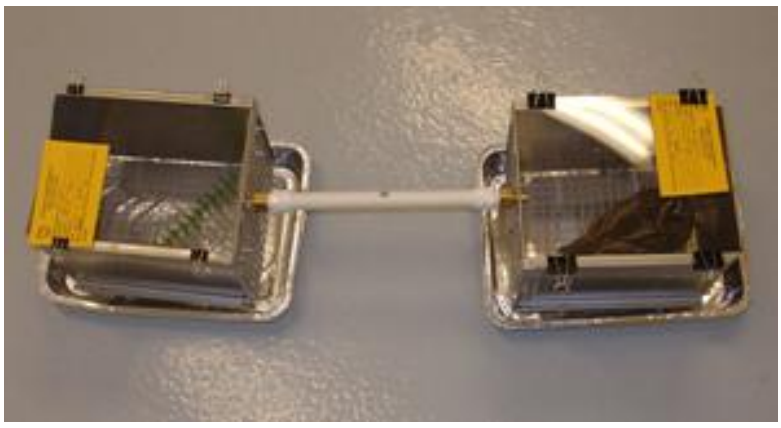

Figure A.1 Cage setup for host preference studies.

Pictured are two cages connected by PVC tubing, where ticks were placed at time zero and allowed to find a host for 24 hours before the tube was removed. This setup was done for every host combination (quail--cotton rat, anole--quail, cotton rat--anole) in triplicate.

Feeding success statistical analyses

A correction was made post-publication of "Observations on Host Preference and Feeding Success of Immature *Amblyomma maculatum* (Acari: Ixodidae)": ANOVAs should have been used instead of the *t*-tests used in the feeding success portion of the experiments. This allows for analysis of all three species (rat, quail, anole), and accounts for the random effect of individuals (n=10 of each species) in the variables tested. The proper analysis is detailed below and was computed using the program R.

```
#Larvae days to engorge
> larvaedaysFit1 <- lme(days ~ species, data=larvaedays, random=~1 | individual)
> summary(larvaedaysFit1)$tTable
                Value Std.Error DF       t-value      p-value
```

(Intercept) -3.354431e-16 0.1356090 52 -2.473605e-15 1.000000e+00

speciesquail 5.396808e+00 0.1589717 52 3.394824e+01 3.684186e-37

speciesrat 5.254009e+00 0.1441485 52 3.644859e+01 1.059295e-38

#Significant differences across species, when considering anoles, but not significant between quail and rats.

#Larvae total engorged

> larvaetotalFit1 <- lme(total ~ species, data=larvaetotal, random=~1 | individual)

> summary(larvaetotalFit1)$tTable

Value Std.Error DF t-value p-value

(Intercept) 4.238047e-16 0.5043808 16 8.402475e-16 1.0000000000

speciesquail 2.750000e+00 0.7565712 16 3.634820e+00 0.0022290633

speciesrat 4.300000e+00 0.7133022 16 6.028301e+00 0.0000175584

#Significant differences across species, when considering anoles, but not significant between quail and rats.

#Nymph weights

> nymphweightFit1 <- lme(weight ~ species, data=nymphweight, random=~1 | individual)

> summary(nymphweightFit1)$tTable

Value Std.Error DF t-value p-value

(Intercept) -1.282946e-18 0.001455736 18 -8.813040e-16 1.000000e+00

speciesquail 1.061002e-02 0.002058722 18 5.153693e+00 6.664932e-05

speciesrat 1.408510e-02 0.002058722 18 6.841672e+00 2.107062e-06

#Significantly different between quail and cotton rats.

#Nymphs days to engorge

> nymphdaysFit1 <- lme(days ~ species, data=nymphdays, random=~1 | individual)

> summary(nymphdaysFit1)$tTable

Value Std.Error DF t-value p-value

(Intercept) 0.5454545 0.3720228 44 1.466186 1.497085e-01

speciesquail 5.8181818 0.4556330 44 12.769449 2.133069e-16

speciesrat 6.7588933 0.4523192 44 14.942750 7.541400e-19

#Significantly different between quail and cotton rats.

#Total number engorged for nymphs

> nymphtotalFit1 <- lme(total ~ species, data=nymphtotal, random=~1 | individual)

> summary(nymphtotalFit1)$tTable

Value Std.Error DF t-value p-value

(Intercept) 6.532798e-16 1.139876 17 5.731150e-16 1.0000000000

speciesquail 7.444444e+00 1.656201 17 4.494893e+00 0.0003192188

speciesrat 5.200000e+00 1.612027 17 3.225752e+00 0.0049645750